The Sec translocase

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The Sec translocase:
Structure-function analysis in protein translocation and membrane protein insertion

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The Sec translocase:
Structure function analysis in protein translocation and membrane protein insertion

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Preface

Towards the end of my Masters degree I really wanted to find out how life was in Europe and combine that with a nice PhD project where I would learn more about protein manipulation. I applied all over the EU in labs where I found interesting projects. However, the combination of Coenie's sales talk, Arnolds quick responses via email, Dutch as a related language to Afrikaans and a very interesting email from Zalán made it clear to me that Groningen will be my new home for a couple of years. I did read about these long winters and lack of sunshine, but I thought to myself, how long can the sun not shine in winter? Two weeks max I thought. Well, we all make some mistakes.

So despite the inclement weather and low quality of meat, do I regret having made the move to the Netherlands for a PhD? Not in the slightest. Its here where I met great friends, the ones which you would like to keep for the rest of your life. It's the place where I met my wife Kamila, and last but not least, I learned a whole lot about membrane proteins. An area I wish to continue to develop myself in as a scientist. I cannot emphasise it enough how invigorating and exciting it is to change your project type and country all in one move! Higly recommended.

Arnold, thank you for accepting me as a PhD student just from our brief discussions over email. I noticed its one of your most endearing qualities as a Professor. You have an excellent manner in having faith in people and giving them loads of free reign in their projects. Your direct approach and front-line thinking made it very interesting and rewarding working under you.

Nico, you were a great copromotor and leader in the group. You were sorely missed after you left, not only for your scientific input, but your whole demeanor. I not only learned how to plan and think of all those extra controls but also how to deal with fellow scientists in a respectful manner.

Bea, I think you must be the rock on which the GBB is resting. A friendly face in the secretariat, and supremely knowledgeable about all organizational things related to the integration of a new foreign PhD. Thank you for all your help.

Greetje, your help in finishing off some final experiments towards the end of my thesis were very welcome! You were always friendly and great to work with.

I am almost afraid of trying to mention all the friends and colleagues who made work and especially life in Groningen so much fun, for fear of leaving out someones name. A poignant quote from G. Randolf to Coenie, Ronan, Francesco, Manfred, Marta WW, Marta P and many more: Truly great friends are hard to find, difficult to leave, and impossible to forget.

My paranimfs Francesco and Manfred. Thank you for supporting me in all the small things around my defense and helping with the organizing of my party and of course cabaret. I know the effort involved and appreciate it!

To Jeanine and Jelger. A big thank you to both of you for checking my Dutch summary for grammatical and other mistakes. A big effort considering the fact that you also prepared to move to the new building.

Aan my familie. Ek is besonders bevoorreg om sulke ongelooflik liefdevolle en ondersteunende familie te hê. Julle vetroue in my en ondersteunende woorde was altyd ‘n riem onder my hart. Daar is nie genoeg woorde om dankie te sê vir alles wat julle vir my gedoen en beteken het in die tyd nie.

Kamila, the love of my life. We walked a long and interesting path together in Groningen. Starting out as friends, we now promote together on the same day as husband and wife. Thank you for all your support and never ending love. Kocham cię!

Francois

Opgedra aan my ouers

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General introduction

D.J.F. du Plessis, N. Nouwen, A.J.M. Driessen

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Summary

The vast majority of protein trafficking across or into the bacterial cytoplasmic membrane occurs via the translocon. The translocon consists of the SecYEG complex that forms an evolutionarily conserved heterotrimeric protein conducting membrane channel that functions in conjunction with a variety of ancillary proteins. For post-translational protein translocation, the translocon interacts with the cytosolic motor protein SecA that drives the ATP-dependent stepwise translocation of unfolded polypeptides across the membrane. For the co-translational integration of membrane proteins, the translocon interacts with ribosome-nascent chain complexes and membrane insertion is coupled to polypeptide chain elongation at the ribosome. These process are assisted by YidC and the SecDF(yajC) complex that transiently interact with the translocon. This review summarizes our current understanding of the structure-function relationship of the translocon and its interactions with ancillary components during protein translocation and membrane protein insertion.

1. Introduction

Many proteins that catalyze essential cell functions are embedded in the cytoplasmic membrane or function on the outside of the cell. These proteins are synthesized at ribosomes in the cytosol and directed to the Sec translocase as the major facilitator in the translocation and insertion of these proteins across or into the inner membrane of prokaryotes, the endoplasmic reticulum membrane in Eukaryotes and the thylakoid membrane of photosynthetic Eukarya [209] (**Fig. 1**). The substrates for the Sec translocase range from very hydrophilic to very hydrophobic proteins, yet all contain a hydrophobic N-terminal region, i.e. a signal sequence for secretory proteins (preproteins) and a membrane anchor signal for inserted inner membrane proteins (IMPs). Translocated proteins are processed by signal peptidase that removes the signal sequence and allows the release and subsequent folding of the mature protein on the outer side of the inner membrane. Lipoproteins are processed by a specific signal peptidase once the cysteine at the +1 position of the mature domain has been lipid modified. This ensures lipid membrane anchoring prior to maturation. The signaling domain of IMPs most often remains associated with the inserted protein.

The Sec translocon is conserved across all three domains of life. Its core consists of a heterotrimeric protein complex designated as SecYEG in Bacteria and Sec61αβγ in Eukaryotes. Ancillary components associated with the translocon provide the energy for translocation and insertion. The translocon can facilitate the movement across or integration of proteins into the membrane in a co-translational or post-translational manner. In Bacteria, the co-translational pathway is mainly employed by inner membrane proteins, while the post-translational pathway is utilized by proteins

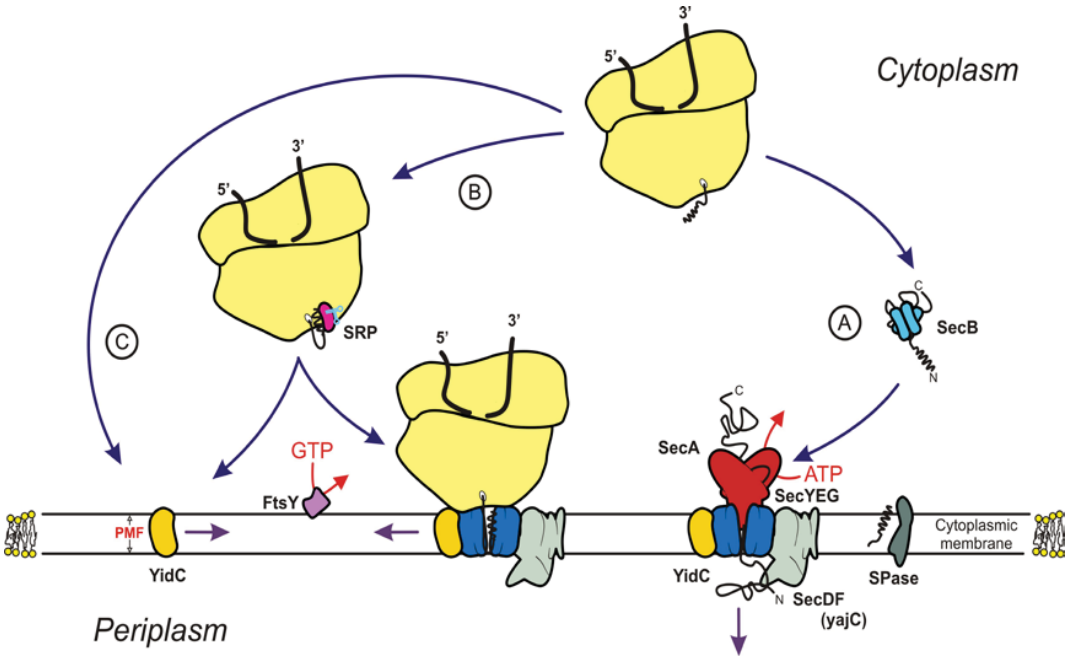

Fig. 1 Schematic representation of protein targeting to the Sec translocase. The bacterial Sec translocon (*blue*) spans the cytoplasmic membrane (CM) and consist of SecY, SecE and SecG. SecA (*red*) acts as the peripherally associated motor protein on the cytoplasmic side. Other ancillary proteins interacting with the translocase include YidC (*yellow*) and the SecDF(yajC) complex (*light grey*). Signal sequences of preproteins are cleaved at the periplasmic face of the membrane by Signal peptidase (SPase). (A) Proteins synthesized at the ribosome (*light yellow*) destined for secretion are mostly post-translationally targeted to the Sec translocase by a targeting sequence which is recognized by the motor protein SecA. Alternatively, the molecular chaperone SecB (*light blue*) binds the preprotein, keeps it in a translocation competent state and targets it to the Sec translocon for translocation. (B) Co-translational targeting of the ribosome with the nascent chain to the translocase complex is attained by the binding of the signal sequence of some preproteins or the signal anchor sequence of membrane proteins by SRP (*pink*) and the SRP receptor FtsY (*purple*). Membrane proteins with large hydrophilic periplasmic domains require the presence of SecA in the insertion process to translocate these domains across the membrane. YidC interacts with TMs as they emerge from the proposed lateral gate of SecYEG; however the importance here is low and not clearly understood. Some proteins such as CyoA and Lep require the translocon, SecA and YidC for its proper insertion; however, limited data exist on how this is achieved precisely. (C) A subset of membrane proteins can insert into the cytoplasmic membrane via YidC after targeting of the ribosome nascent chain to YidC. Conflicting evidence exist for the involvement of the SRP pathway. Some studies indicated the targeting of the ribosome nascent chain to YidC via the SRP pathway, whereas SRP depletion studies showed no effect on membrane insertion suggesting a direct targeting of the ribosome nascent chain to YidC.

that are secreted across the membrane [264]. The selection step for either pathway lies at an early stage of translation once the nascent chain emerges from the ribosomal exit tunnel [183,266]. Ligand crowding at the exit tunnel allows signal recognition particle (SRP) to be the first to interact with the ribosome nascent chain (RNC) [77]. If the emerging signal sequence displays a high level of hydrophobicity [289] and helicity

[33], SRP binds the RNC tightly and it has been suggested that this binding reaction results in a pausing or slow-down of translation (See also below). The entire SRP-RNC complex is then targeted to the membrane-associated signal-particle receptor (FtsY) that itself is bound to a competent translocon [183]. SRP and FtsY form a heterodimeric complex and this stimulates GTP hydrolysis on both proteins, whereupon the RNC is transferred to the translocon with the ribosomal exit tunnel in close proximity to the translocon pore [23]. Polypeptide chain elongation at the ribosome provides the energy for the co-translational insertion of membrane proteins.

When a signal sequence emerging from the ribosome does not display a high level of hydrophobicity, it is bound by trigger factor which shields it for further binding by SRP [77]. Next, the polypeptide is translated through its full length by the ribosome in the cytosol. In a subset of Bacteria (mostly Proteobacteria), the newly synthesized preprotein is maintained in an unfolded state by the cytosolic molecular chaperone SecB as firmly demonstrated by recent single molecule measurements [19] {For a review on SecB see [82]}. Next, the SecB-preprotein binary complex is targeted to the translocon where SecB binds to the ATPase motor protein SecA. After the release of SecB, and the transfer of the preprotein to SecA, translocation is initiated at the expense of ATP [236]. In contrast to bacterial protein translocation that is strictly dependent on SecA, translocation in the Eukaryotic ER can also occur co-translationally [90]. During post-translational translocation in Eukaryotes, the ER luminal protein BiP provides the energy for translocation at the anterior or exit site of the translocon as opposed to SecA which is found at the interior face of the translocon [202]. For Archaea, post-translational translocation has been suggested to occur, however, Archaea lack a SecA homologue. Although they contain BiP homologues,

i.e., Hsp70 proteins, these chaperones reside in the cytosol and thus cannot bind at the exit site of the translocon like in Eukaryotes. Therefore, a major unresolved question is how post-translational translocation in Archaea is energized. IMPs with large periplasmic domains represent a special class. These proteins insert into the membrane in a co-translational manner, but require SecA for the translocation of their large polar domains. This involves a mechanism in which the ribosome while actively engaged in the synthesis of a polar domain must be released from the translocon in order to allow SecA binding.

To date, various requirements and conditions have been described for the translocation of proteins and the insertion of membrane proteins. Particularly, a great advancement in the understanding of these processes has come with the development of biochemical *in vitro* assays to determine the minimal components required. There is no other protein translocation system to date that has been studied at such advanced level, including crude membrane systems up to liposomes reconstituted with the purified components performing the key activities of the translocon [34]. Here, we will give an overview of the factors involved in translocation of polar polypeptide domains across the membrane, and the insertion of apolar polypeptide domains into the membrane. The focus is on the role of the Sec translocase acting as a multipurpose device that facilitates these two seemingly opposing activities. The term translocon is used to indicate the protein conducting channel while the term translocase includes the translocon with one of its energy supplying ligands, i.e., in Bacteria, SecA or the ribosome.

2. The translocase – components and structure

2.1. Organization of the translocon

The Sec translocon exhibits the distinct ability to both translocate substrates across a membrane as well as to insert them into the membrane laterally. Its functional properties have been studied in great detail. The translocon in Bacteria consists of three proteins, namely SecY, SecE and SecG [34]. The Sec61p of the endoplasmatic reticulum (ER) is homologous to the SecYEG complex with an identical trimeric arrangement of Sec61α (homologous to SecY), Sec61β and Sec61γ (homologous to SecE) [91]. Sec61β is not homologous to SecG in either structure or function. Rather, Sec61β has been implicated in acting as a guanine nucleotide exchange factor for the SRP receptor [104]. SecG stimulates the activity of SecYE by attenuating the SecA activity [185,186,188,280]. In the yeast ER (*Saccharomyces cerevisiae*) two homologous Sec systems exist. One Sec61p complex consisting of Sec61p (Sec61α), Sbh1p (Sec61β) and Sss1p (Sec61γ) as subunits and a second complex consisting of Ssh1p (Sec61α) and Sbh2p (Sec61β) [83]. While the components of the two translocons can interact, the Ssh1p complex is not essential for viability and has been shown to be involved in co-translational protein translocation only [83,225,298]. The Sec61p complex can interact with the Sec62p complex that consists of three membrane subunits Sec62, Sec63 and Sec70/72 which are involved in the BiP-mediated post-translational translocation of preproteins across the endoplasmic reticulum membrane [119,202,207]. In the thylakoid membranes of chloroplasts homologues to SecY and SecE have been characterized that together with SecA mediate the translocation of preproteins across the thylakoid membrane

[154,176,233,234,238]. SecY homologues have also been found in cyanelles [60,84,288], cyanobacteria [177] and certain mitochondria of primitive protists [35]. In Archaea, homologues for the translocon exist as SecY, Sec61β and SecE with notably the absence of a SecA or possible BiP homologue that could be involved in post-translational translocation [10,12,129,137]. The archaeal subunits are more closely related to those of the Eukaryotes than the bacterial ones [76].

SecY has a molecular mass of 48 kDa, spans the membrane ten times in an α-helical configuration and is highly hydrophobic. It is the largest component of the translocon and essential for viability and translocation. In *E. coli*, SecE is a small integral membrane protein of 14 kDa with three predicted transmembrane segments (TMs). Other Bacteria have SecE proteins with a single TM that is homologous to the third TM of the *E. coli* protein [173]. Despite its small size, SecE is essential for viability and translocation. Only the third TM of the *E. coli* SecE is required for a fully functional translocon corresponding with the observation that most SecE homologs consist only of a single TM [49,221]. SecY and SecE form a stoichiometric complex, and in the absence of SecE, SecY is unstable and readily degraded by FtsH [133]. SecY seems to be toxic to cells when not associated with SecE as the overproduction of SecY in a conditional lethal FtsH mutant background leads to the inhibition of cell growth as well as protein export [133]. SecG is a 12 kDa protein containing two TMSs. While not essential for viability or translocation, SecG displays some remarkable biochemical properties. SecG has been found *in vitro* to stimulate preprotein translocation, particularly at low temperatures [185] or when the proton-motive-force (PMF) is reduced [95]. SecG has been hypothesized to facilitate the binding and insertion of SecA into the translocon by undergoing transient topological inversions during protein translocation. This hypothesis is based on biochemical

studies by Tokuda and coworkers that showed an altered proteolysis pattern of SecG [188] as well as a change in accessibility of loop residues for chemical modifications [174] during protein translocation. However, another study showed that a topologically fixed SecG is fully functional in protein translocation [280]. No evidence has been found for a physical interaction between SecE and SecG, and SecG has been found to bind SecY in the absence of SecE [110], thereby weakly improving the stability of SecY [187] and of the SecYE complex [110].

2.2. Structure of the translocon

The first high-resolution structure reported was of the archaeal SecYEβ complex of *Methanocaldococcus jannaschii* [270]. The structure has provided many new insights in the structure-function relationship of the translocon and the possible mechanism of channel opening (**Fig. 2**). Superimposition of the crystal structure onto the three-dimensional reconstruction of the *E. coli* SecYEG based on electron cryo electronmicroscopic imaging of two-dimensional crystals [32] revealed that the two complexes differ only slightly in conformation [30]. The ten TMs of the main subunit SecY are arranged like a clamshell in which the two halves TMs 1-5 and TMs 6-10 are hinged at the cytoplasmic loop between TM5 and TM6 (**Fig. 2**B). The SecE protein embraces the two SecY halves at the so-called “back” end of the translocon with its TM crossing through the membrane diagonally. The surface exposed amphipathic helix of SecE lies flat onto the cytoplasmic side of the cytoplasmic membrane [287]. The SecY channel has the shape of an hourglass, with at its centre a constriction that narrows the channel to a few Ångstroms suggesting that

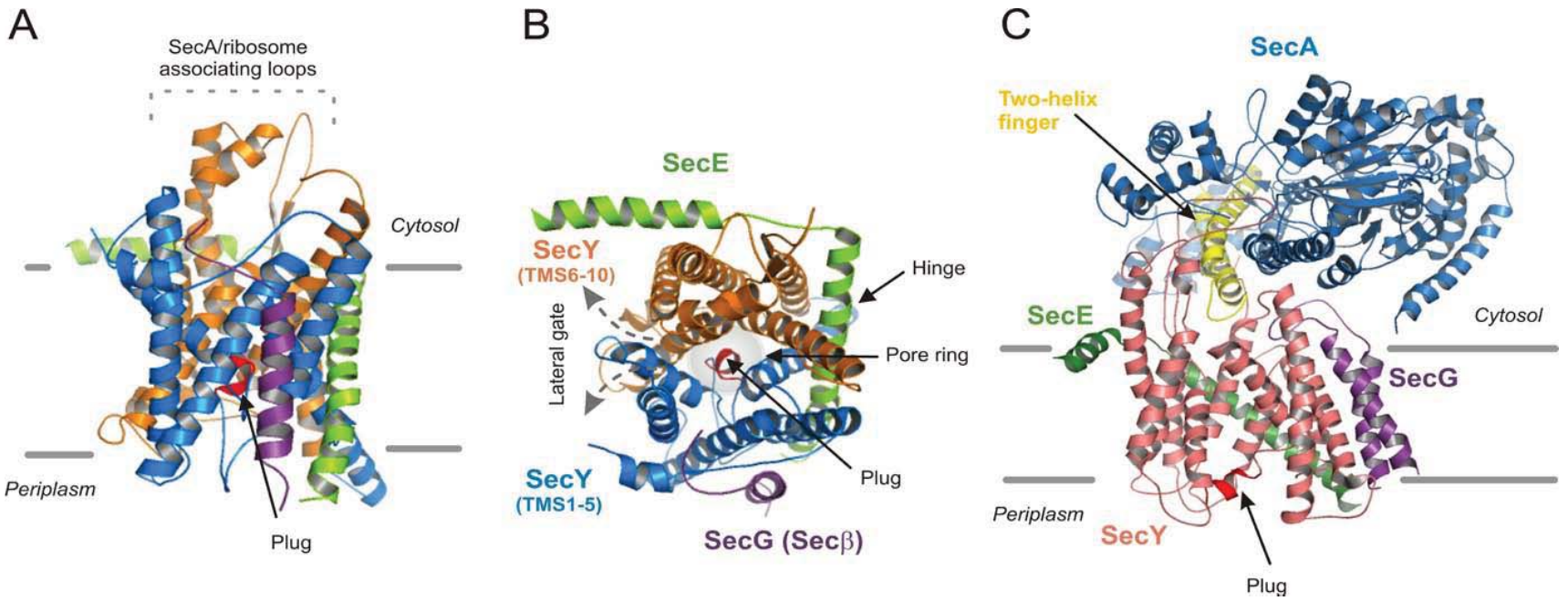

Fig. 2 Structure of the Sec translocase. (A) Side view and top view (cytosolic face) (B) of the crystal structure obtained from SecYEβ of *M. jannaschii* based on coordinates deposited at the Protein Data Bank as 1RHZ [270]. The hourglass shaped translocon consists of SecY (Sec61α) as the core of the translocon, SecE (Sec61γ) that embraces SecY in a supportive manner and SecG (Sec61β) which is peripherally bound to SecY. At the centre of the SecY channel a pore ring, consisting of hydrophobic amino acids, forms a constriction. A small alpha-helix (TM2a) that folds back as a re-entry helix acts as a plug (*red*) from the periplasmic side and together with the pore ring serves to close off the channel and prevent ion leakage. The central pore formed by SecY is arranged in a clamshell like arrangement with TMS 1-5 (*blue*) and TMS 6-10 (*orange*) forming the two halves. The clamshell is hinged at the back towards SecE. It has been proposed that the front of the clamshell at the intersection between TM7 and TM2b can open up and act as a gate to laterally release TMs into the lipid bilayer. Proteins that are translocated bind with their signal sequence at the TM2b and TM7 interface leading to the widening of the central pore and destabilization of the plug. As a consequence the central pore opens to accept proteins for translocation across the lipid bilayer in a concerted effort together with SecA insertion [270]. (C) Side view of the crystal structure for the SecA/SecYEG complex from the Gram-negative Bacteria *T. maritima* based on coordinates deposited at the Protein Data Bank as 3DIN [311]. The binding of SecA (*blue*) to SecYEG (*pink, green* and *purple*) induces a conformational change in SecYEG whereby the lateral gate of SecYEG opens up to the lipid interface, the plug (*red*) moves away from the centre of the pore and SecA inserts a two-finger helix (*yellow*) which has been proposed to move up and down the channel with cycles of ATP hydrolysis.

the *M. jannaschii* structure represents the closed conformation of the translocon. The centre of this constriction contains six hydrophobic amino acid residues which have their hydrophobic side chains directed towards the centre of the channel. Proteins are thought to pass the translocon through the central pore and the hydrophobic residues within the constriction have been proposed to form a seal around the translocating protein thereby maintaining the permeability barrier of the membrane during translocation. At the cytoplasmic side, the water-filled channel has an opening of 20 to 25 Å where SecA, the ribosome and the polypeptide likely interact with the channel. At the external or periplasmic face of the membrane, TM2a forms a re-entry loop that folds back in the outer funnel to block the channel. TM2a is also referred to as the "plug" domain. A previous crosslinking study [99] has led to the suggestion that upon binding of a signal sequence to the translocon, the plug is displaced allowing preprotein translocation to occur [270]. Signal sequences of preproteins can be crosslinked to the TM2 and TM7 [206,207,295]. These two helices are at the 'front' of the translocon, and it has been proposed that insertion of the signal sequence into the TM2/TM7 interface induces a separation of the two halves of SecY which would further facilitate the displacement of the plug from its central position. A recent molecular dynamics study has shown that the opening the channel by this mechanism creates an opening that is large enough for the passage of unfolded and even larger α-helical domains in proteins [255]. The opening between TM2 and TM7 would also allow the lateral partitioning of TMs and signal sequences into the lipid bilayer and thereby expected to play a very important role in the insertion of membrane proteins via the Sec translocon. For this reason, this region was termed the 'lateral gate'. Based on a crosslinking study [99], it has been suggested that in the open state, the plug may

completely vacate the central channel and move to a position close to the C-terminus of SecE [270]. Besides liberating the exit-site, the hydrophobic surface of the plug domain may guide the unfolded protein towards the periplasm [28] and in combination with the hydrophobic constriction ring involved in sealing of the preprotein-translocon junction [270].

In recent years, two independent studies presented the structure of the Sec translocon in a 'pre-open' state. The structure of the *Thermus thermophilus* SecYE complex is with a Fab fragment bound to the cytoplasmic loops C4 and C5 of SecY [259]. This Fab fragment was proposed to induce a conformation that would correspond to the SecA bound state of SecYEG. Herein, TM6, TM8 and TM9 were displaced by 10 Å relative to the *M. jannaschii* SecYEβ structure. The structure of the *Thermotoga maritima* SecYEG translocon with bound SecA (**Fig. 2**C) was solved at a resolution of 4.5 Å [311]. The SecA protein had bound ADP-berrylium fluoride, which is a transition state analog of ATP hydrolysis. In this structure, the tightly bound SecA causes the lateral gate of SecY between TM2 and TM7 to open, with the TM2a plug domain moving away in the direction of the periplasmic side of the translocon. Furthermore, a two-helix finger of SecA was found to insert near the "front" entrance of the channel (See next section). Again this structure appears to adopt a 'pre-open' state. Importantly, the structure indicates that lateral gate opening and SecA function might be allosterically linked. Indeed, biochemical studies showed that the opening of the lateral gate of SecYEG is essential for SecA-mediated protein translocation and linked to the activation of the SecA ATPase activity [67]. In the 'pre-open' state of SecYEG, the plug domain moves towards the C-terminus of TM7 but still closes the central pore. This is different from the proposed 'open' state where the plug domain is suggested to be completely moved towards the C-terminal tip of

SecE [99,270]. A recent crosslinking study demonstrated that only a small displacement of the plug occurs during channel opening [161]. Interestingly, a molecular dynamics simulation [308] suggests that the plug domain may function as a kind of 'ruler' that senses the polarity of the incoming polypeptide. For polar polypeptide domains that need to cross the channel, plug displacement would result in the formation of a vectorial aqueous pore. In contrast, an apolar polypeptide domain that needs to insert into the membrane would not induce such plug displacement. However, a true function of the plug as ruler remains to be demonstrated biochemically.

2.3. SecA, a preprotein-stimulated translocation ATPase

SecA functions as a motor protein both in protein translocation as well as in the translocation of hydrophilic domains of membrane proteins across the membrane. SecA associates with SecYEG, and this interaction involves the major cytosolic loops of SecY [125,165,226]. The interaction of SecA with the translocon occurs at a much higher affinity than the interaction with preproteins in the cytoplasm [92,100]. In addition, SecA interacts with anionic phospholipid headgroups at a low affinity [159]. The structures of SecA proteins has been characterized by X-ray crystallography in great detail employing proteins derived from various organisms [115,190,200,203,245,285,310,312]. Most of these structures display SecA packed as a dimer (**Fig. 3**) with an antiparallel orientation except for the *T. thermophilus* SecA that was crystallized as a parallel dimer [285]. SecA belongs to the superfamily 2 DExH/D proteins [143], and contains a motif that is also found in DNA/RNA helicases. The conserved DEAD helicase motor, made up by the two nucleotide

binding subdomains (NBF1 and NBF2), is the site for ATP binding and hydrolysis (**Fig. 3**).

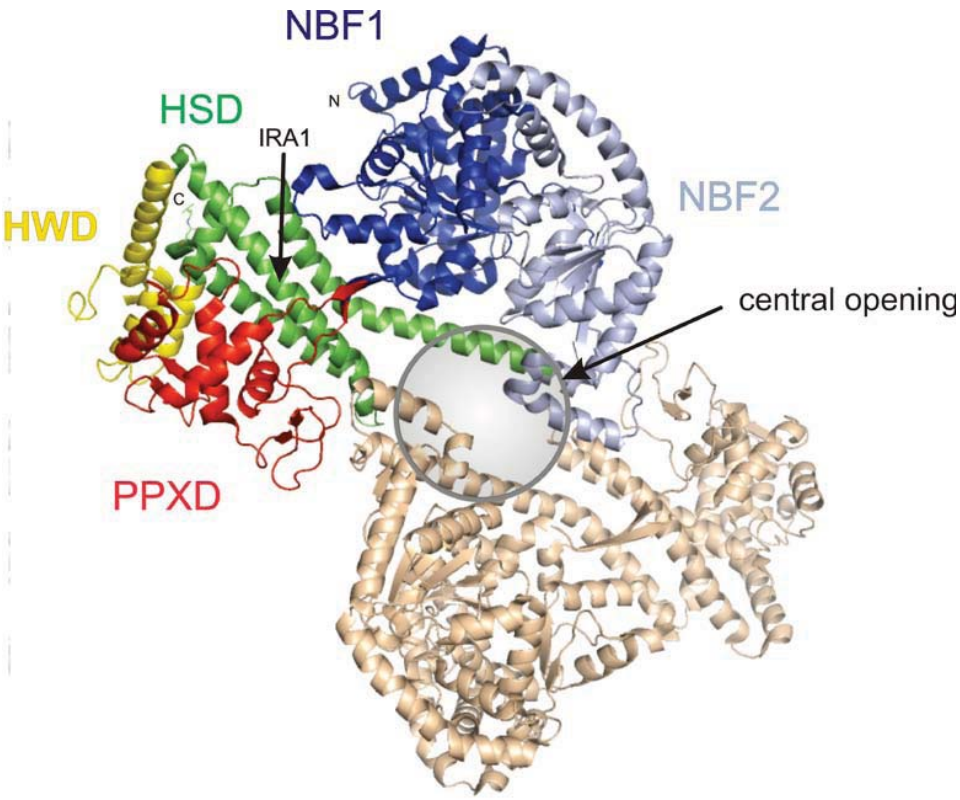

Fig. 3 Structure of SecA, the motor protein of the Sec translocase. Graphic representation of the *Mycobacterium tuberculosis* SecA based on coordinates as deposited at the Protein Data Bank as 1NKT [245]. The antiparallel crystal structure as obtained is displayed here with the second SecA protomer in its antiparallel arrangement in beige. Also indicated is the formation of a central pore at the centre of the dimer. Nucleotide binding folds 1 and 2 (NBF1, NBF2) are shown in dark blue and light blue respectively. The preprotein crosslinking domain (PPXD) is shown in red, while α-helical scaffold domain (HSD) is shown in green. Finally the α-helical wing domain (HWD) is shown in yellow. The C-terminal linker domain (CTL) was not resolved in this structure. The intramolecular region of ATP hydrolysis 1 (IRA1) which is responsible for the hydrolysis of ATP at NBF1 is indicated.

The ATP hydrolysis cycle drives conformational changes in the motor domain [88,115,249] that are transferred to the helical wing domain (HWD) and the

preprotein cross-linking domain (PPXD) [88,249]. SecA interacts with preproteins via its PPXD domain [136,204]. The helical scaffold domain (HSD) lies at the centre of the protomer, where one of the α-helices interacts with all other subdomains of SecA. The C-terminal linker domain of SecA (CTL) has been shown to constitute a zinc finger and shown to be involved in both SecB binding and the interaction with phospholipids [31,80].

Several functionally important regions in the SecA protomer have been defined. Within the HSD domain a helix-loop-helix structure that contacts both the PPXD and NBF2 subdomains have been identified to act as a global regulator of ATP turnover. This region is indicated as the intramolecular regulator of ATP hydrolysis 1 (IRA1) (**Fig. 3**). This domain most likely prevents promiscuous ATP hydrolysis in the cytosol, as a deletion or mutations in this domain lead to an ATPase activity that is uncoupled from preprotein interaction [127]. A conserved salt-bridge known as Gate 1 controls the opening and closing of the nucleotide binding groove in concert with the binding signal observed at the PPXD domain [126]. However, this mechanism seems to be only active once SecA is bound to SecYEG thereby leading to a synchronized preprotein binding and release cycle coupled to ATP hydrolysis and resulting in the step-wise translocation of the preprotein across the membrane [236,281].

Experimental evidence to date favors a model where SecA inserts itself partially into the translocon during protein translocation [67,75,78]. This inserting domain corresponds to a two-helix finger of SecA that binds and interacts with the preprotein substrate during translocation [78]. It was proposed that the two-helix finger may drive translocation by insertion into the cytosolic funnel-like opening. Crosslinking data indicate that SecA captures the preprotein in a clamp-like manner,

whereupon the preprotein may move through the clamp as an extended protein and enter the SecYEG translocon [18]. This clamp-like structure has recently been resolved for the *Bacillus subtilis* SecA in complex with a peptide [312]. Similarly, the signal peptide binding domain within *E coli* SecA has been visualized utilizing FRET measurements [11]. The signal peptide binding domain within SecA seems to comprise part of the PPXD as well as regions from NBF1 and the HSD. From these two studies it has become clear that preprotein binding and translocation in SecA occurs at a multi-domain interface further indicating the high flexibility of SecA during protein translocation.

With regards to the role of SecA in insertion of membrane proteins, it has been shown that inner membrane proteins with periplasmic domains larger than 60 amino acids require SecA for their correct and complete insertion [6]. Membrane proteins with smaller periplasmic loops do not require SecA or SecG for their insertion [139]. One can envisage a membrane protein with periplasmic loops of varying size that would intermittently require SecA. How this is achieved together with co-translational membrane protein insertion involving the ribosome, is not understood. It has been suggested that SecA and the ribosome bind the translocon simultaneously during co-translational translocation [313], but since SecA and the ribosome bind to overlapping binding sites of the SecY protein it is not clear how simultaneous binding might occur. Moreover, in the cytosol SecA also binds directly to ribosomes [128] The exact role of SecA in membrane protein insertion, and in particular the dynamics of the interaction of SecA with the ribosome and translocon requires further investigation.

2.4. YidC and SecDF(yajC)

SecDFyajC is a membrane protein complex that associates in a transient fashion with the Sec translocon [70] and stimulates preprotein translocation. Although SecD and SecF are not essential, their inactivation in *E. coli* results in a severe pleiotropic protein secretion defect as well as a severe growth inhibition [208]. Interestingly, some Bacteria such as *Lactococcus lactis* lack a SecDF complex and the introduction of a heterologous SecDF protein results in improved preprotein translocation [191]. Mutations in SecDF may result in a cold-sensitive growth phenotype. SecDF has initially been implicated in the cycling of SecA during preprotein translocation [71] whereas its role in membrane protein insertion is less clear [42] and possibly even indirect as depletion of SecDF affects the SecG levels *in vivo* [130]. SecDF have also been implicated in PMF-dependent translocation [71], but even in de absence of SecDF, translocation remains PMF-dependent [197]. Recently, a preliminary X-ray diffraction study has been reported on the SecDF protein from *T. thermophilus* [260]. Future structural studies will likely shed more light on the role of this mysterious subunit of the translocase. Especially, the role of SecDF in membrane protein biogenesis warrants further investigation.

In the last decade, another essential protein has been identified that interacts with the translocon but that appears to fulfill a specific role in membrane protein insertion (**Fig. 1**). This protein, YidC, is a member of the Oxa family of membrane proteins consisting of YidC in Bacteria [229], Oxa1p in mitochondria and Alb3 in the thylakoid membranes of chloroplasts [151]. YidC plays an essential role in the insertion of a subset of membrane proteins via the translocon [68,141,268], while it also has been shown to crosslink to membrane proteins exiting the translocon that are

not dependent on YidC for their insertion [20,111,240,265]. Interestingly, YidC has also been implicated in the insertion of SecE [306]. Cross-linking studies suggest that YidC transiently interacts with the translocon during the insertion of IMPs and this interaction may involve SecD and SecF through a heterotetrameric YidC-SecDFyajC complex [195]. Importantly, YidC is also able to act as an insertase on its own [213,214,229,274]. To date, the YidC substrates identified are rather limited, although most appear to be membrane subunits of large respiratory complexes, such as CyoA of the bo_3 cytochrome oxidase, subunits a and c of the F_1F_o-ATPase, and NuoK of the NADH dehydrogenase I. Phage M13 and Pf3 coat proteins also require YidC for their insertion [41,229,230]. Several membrane proteins depend on YidC for folding rather than membrane insertion, while the observation that the entire F_1F_o-ATPase complex can be co-purified with YidC homologs in *Bacillus subtilis* [228] suggests that YidC fulfils additional roles in folding and complex assembly. For a recent review describing the role of YidC in inserting and assembling subunits of large respiratory complexes see [142] and a review on the role of YidC homologues in Gram positive Bacteria as well as Archaea see [307]. Also, a recent review discusses the biogenesis of respiratory chain complexes with a focus on the role of YidC in these processes [215].

The structure of YidC is unknown. Remarkably, the large periplasmic domain does not contribute significantly to the function of YidC [121]. This domain has a β-fold sandwich structure with a possible substrate binding cleft [198,222]. Interestingly, while the five terminal TMs are essential for YidC activity, systematic mutagenesis of residues in TM2, TM3 and TM6 as well as swapping TM4 and TM5 with unrelated TMs proved to have little effect on YidC activity [121]. Thus, it seems that YidC is rather invariant for mutagenesis, indicating a role as an insertion platform

rather than an active insertase. On the other hand, YidC seems to undergo conformational changes upon substrate binding [299]. A subset of Bacterial YidC homologs contains a C-terminal extension that shows some homology to the C-terminal region of Oxa1p that has been implicated in ribosome binding [86]. Various Bacteria contain more than one YidC homolog that may differ in the presence of this C-terminal extension. Possibly, there is a functional differentiation of YidC-like proteins that act in co-translational membrane protein insertion requiring an interaction with the ribosome, and the insertases that may act post-translationally. A recent cryo-EM study proposed that YidC, in association with a translating ribosome forms a dimeric pore [140]. The interface of interaction may involve TM2 and TM3. On blue-native gels, YidC migrates as a monomer or dimer, while Oxa1 is found as a tetramer [181,275]. It would be interesting to investigate the oligomeric state of YidC as it interacts with the SecYEG translocon, especially in context of its larger structure that it seems to form with SecDF(yajC). In this respect, a structural analysis of YidC will be essential for our understanding of this seemingly promiscuous yet essential protein.

3. Oligomeric state of the translocon

The oligomeric state of the translocon and its ancillary proteins during protein translocation and membrane protein insertion is a controversial topic. As many of these components are interacting, we will give a brief overview of the discussion on the oligomeric states of SecA and the translocon.

3.1. Monomeric versus dimeric SecA

SecA can be found in the cell either in a soluble or membrane bound form. Cytosolic SecA exists in a dynamic equilibrium between a monomeric and dimeric state [300] with a dissociation constant of around 1 nM (Ilja Kusters, unpublished results). Since the cellular concentration of SecA is close to 8 μM [4], the dimer will be the predominant species in the cell. High salt and detergent promote dimer dissociation, whereas the equilibrium is also affected by translocation ligands such as synthetic signal peptides and nucleotides. Although these studies indicate a fragile monomer-dimer equilibrium for SecA in solution, the oligomeric state of SecA bound to the SecYEG complex has been a major topic of controversy. In detergent solution using methods such as native gel-electrophoresis and gel-filtration studies, both the monomeric and dimeric SecA have been shown to bind SecYEG [24,69,262]. By chemical crosslinking and surface plasmon resonance (SPR) analysis [53,123], it appears that SecA while bound to SecYEG remains dimeric throughout the translocation reaction. At very low concentration, SecA can also be crosslinked as a monomer to SecYEG [199] but under those conditions, the system is essentially inactive. By means of mutagenesis and truncation, the monomer-dimer equilibrium can be shifted to yield mostly the monomeric species. However, such mutants are severely compromised in preprotein translocation [123,199,220]. Another study investigated the functional oligomeric state of heterodimeric SecA consisting of combinations of inactive and active SecA monomers [64]. Here, heterodimers were found to be completely inactive, lending strong support that SecA functions as a dimer.

Crystallization of different SecA proteins resulted in crystals in which in most cases SecA was present as a dimer in an anti-parallel orientation as shown in **Fig. 3**. Only one SecA dimer, SecA from *Thermus thermophilus,* has been crystallized in a parallel dimer orientation [285]. However, the recent crystal structure of monomeric SecA bound to SecYEG [311] demonstrates that a stable complex can be formed between monomeric SecA and SecYEG. Since the crystallization was performed in detergent solution at high salt concentration, both conditions that cause a dissociation of the SecA dimer, the obtained crystal structure does not exclude the possibility that the SecA dimer is the active state during protein translocation as suggested by functional studies. While many of the crystallized SecA dimers also have been observed as dimers using other biochemical approaches [59,199,310], it is still unclear if the SecA dimer is a physiologically relevant state. Suggested roles for the dimeric form of SecA are: 1) a cytosolic chaperone that guides preproteins to the translocon, 2) the inactive state of the motor protein, and 3) the physiological relevant conformation for interacting with the translocon. For a recent overview on the state of the art in the SecA monomer versus dimer hypotheses see [231].

3.2. Oligomeric state of SecYEG

Also with respect to the oligomeric state of SecYEG, a considerable controversy exists in the field. The oligomeric state of detergent-solubilized SecYEG in the absence of any ligand revealed, similar to SecA, that the translocon can be found in a dynamic equilibrium between monomers, dimers and even higher order oligomers. These different oligomeric states have been observed using a variety of techniques such as density centrifugation [30], analytical ultracentrifugation [44],

native gel-electrophoresis [24], gel filtration [262] as well as negative stain electron microscopy [97,164,167]. Also, higher order oligomers were found with SecYEG reconstituted into proteliposomes [235]. Functional studies with a tandem SecY-SecY fusion construct that yielded covalently linked SecYEG dimers suggests a functional asymmetry in the translocase with one of the SecYEG channels acting as a binding frame for SecA and the other channel acting as a translocation pore [201]. Remarkably, this dimeric SecYEG orientation is not supported by a structural analysis of the monomeric SecA-SecYEG complex that was suggested to suffice for protein translocation [311]. Another study suggests that the phospholipid cardiolipin fulfils a

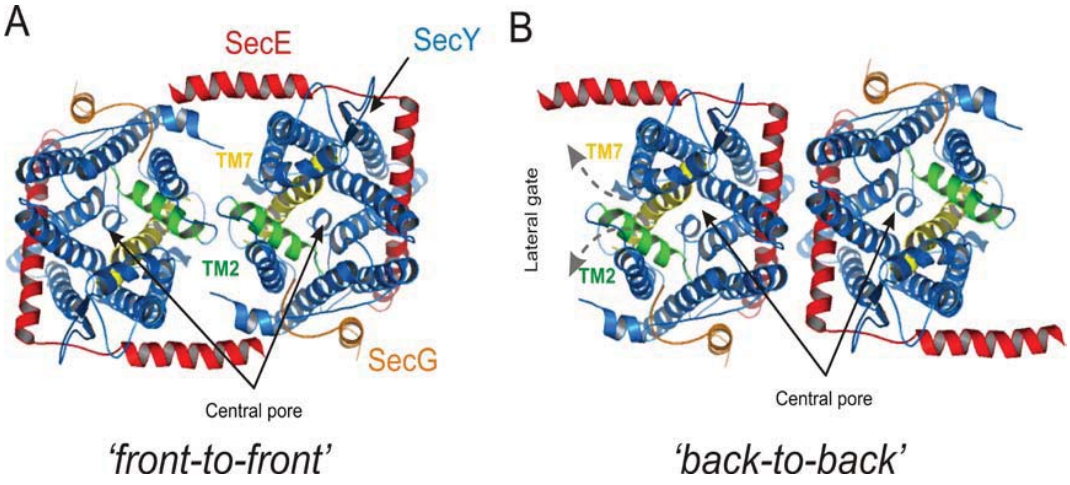

Fig. 4 Schematic representations of the proposed oligomeric states of the Sec translocon. (A) The front-to-front model for the Sec translocase with the lateral gates formed by TM2 (*green*) and TM7 (*yellow*) opposing each other. SecE (*red*) braces the two translocons on either side. It has been proposed that in this state the two SecY channels (*blue*) can from a single consoled pore [170]. (B) Some biochemical data has suggested a back-to-back arrangement for two SecYEG translocons. In this instance, the two translocons are aligned with the large TM of SecE. Both translocons can act independently of each other with membrane protein insertion and protein translocation. Indicated are the locations of the central pores as well as the TM2/TM7 lateral gate.

crucial role in driving the dimerization of SecYEG. It was suggested that monomeric SecA is bound to both protomers of the SecYEG dimer wherein one of the protomers only functions as a supporting platform [89]. It is difficult to access the functional

significance of these observations as cardiolipin is not required for protein translocation and viability.

Interestingly, in other studies SecA has been found to bind only to dimeric SecYEG [69,262]. It has been reported that the binding of SecA to SecYEG shifts the equilibrium of monomeric SecYEG towards the oligomeric state [164,235]. Also, covalently-linked SecYEG dimers [69,201] as well as disulfide crosslinked translocons [279] were all found to be active in protein translocation. Electron microscopy (EM) has also been utilized to determine the oligomeric state of SecYEG and the Sec61p complex. Earlier low resolution EM images, showed ring-like structures of translocons associated with ribosomes or SecA and these were suggested to be oligomeric states of the translocon, such as dimers, trimers or tetramers [23,97,164,167,171]. Interestingly, a more recent higher resolution analysis indicates the presence of a monomeric SecYEG to be bound to ribosomes charged with a nascent membrane protein [22].

Although current evidence suggests that a monomeric SecYEG suffices for protein translocation, an interesting question with the alternatively proposed SecYEG dimer is how it is oriented. Currently, two models for the orientation of the dimeric translocon exists (**Fig. 4**), the 'front-to-front' and 'back-to-back' orientation. In the 'front-to-front' orientation, the lateral gates of the two SecYEG complexes are facing each other. In this orientation, there is the possibility that the two channels fuse to form a consolidated channel. On the other hand, in the 'back-to-back' orientation, the two SecYEG complexes are in contact via the transmembrane segment of SecE that embraces the SecY subunit as a clamp. A cryo-EM study on a ribosome-nascent chain-SecYEG proposed that the two SecYEG complex in the dimer are in a 'front-to-front' manner [171] (**Fig. 4**A). It was suggested that the ribosome senses at an early

stage the presence of a nascent preprotein or IMP in the tunnel, and that this results in conformational changes within the ribosome that are transduced to the translocon [169]. This may result in an assembly of a dimer and even induce the opening of the channel. It was suggested that one channel of the dimer functions as an exit site for the inserting transmembrane domain whereas the other channel acts as the translocation pore [170]. Interestingly, in a biochemical study various single cysteine residues introduced in the "front" of the translocon resulted in a very efficient crosslinking of SecYEG into a dimeric complex [279]. However, no further biochemical or other cryo-EM experiments have been put forward to test the front-to-front model. Again, higher resolution structures from cryo-EM are required so that TMs can be assigned in the structure unambiguously, but a recent cryo-EM study of the ribosome-bound Sec61p complex suggests the presence of a monomeric translocon excluding the dimer hypothesis [22].

The 'back-to-back' model (**Fig. 4**B) for the translocon has been suggested on the basis of a very effective crosslinking of unique cysteine positions between neighboring SecE proteins [131,286]. With this SecE-SecE crosslink [131] translocation is strongly inhibited, suggesting either that this orientation of the dimer is not compatible with protein translocation, or that some form of flexibility is required that is impaired by the introduced crosslink. Interestingly, an 8 Å resolution structure of the *E. coli* SecYEG, as determined by cryo-EM from two-dimensional crystals [32,44] showed two SecYEG translocons in a twofold symmetry axis at the third TM of the SecE protein in close contact. An atomic homology model of the 'back-to-back' crystal structure [32,44] was build by incorporating the atomic structure of the *M. jannaschii* SecYEG [30,270]. The recent X-ray crystal structure of SecYEG from the Gram-negative *T. maritima* bound to SecA (**Fig. 2**C) shows a tight

interaction between SecA and a single SecYEG complex. An extensive crosslinking analysis of the sites of interaction between SecA and SecY included many positions on SecA that remained unaccounted for in the monomeric SecA-SecYEG structure [123,163]. Although docking attempts with a second SecYEG complex could not provide evidence for such sites of interaction [311], it was argued that the most plausible orientation in the proposed SecYEG dimer is the 'back-to-back' orientation. However, in a recent docking study it was argued that major domain movements in the SecA protein will allow for 'back-to-back' orientation of the SecYEG dimer that takes the observed crosslinks into account [89].

4. Ribosome targeting by SRP

The targeting of ribosome-nascent chain complexes (RNCs) to the translocon is universally conserved over all domains of life [210]. The signal recognition protein (SRP) binds the RNC once a hydrophobic signal sequence or transmembrane segment has emerged from an actively translating ribosome. At the membrane, the complex is recognized by the SRP receptor FtsY (SR) whereupon a heterodimeric SRP-SR complex is formed. Upon binding and hydrolysis of GTP, SRP is released and the RNC is transferred to the translocon. Under those circumstances, continued translation is coupled to the insertion of membrane proteins and in Eukaryotes also in the translocation of proteins (**Fig. 1**).

In *E. coli*, SRP is a ribonucleoprotein with a conserved structure. The protein component is known as Ffh (fifty-four homologue), which is associated with the 4.5S RNA [212,223]. Ffh is homologous to SRP54 in Eukaryotes while 4.5S RNA is partly homologous to eukaryotic 7SL RNA. The eukaryotic SRP has a more complicated

domain organization as compared to the bacterial SRP (see for review [93]). In Bacteria, the SRP system is mainly involved in the targeting of IMPs to the translocon, while in the endoplasmic reticulum (ER) of Eukaryotes the SRP targets both IMP and preproteins [49]. SRP is a multidomain protein with an intrinsically unfolded acidic (A-) domain, a conserved GTPase (G-) domain and a signal sequence binding M-domain [93]. The bacterial SRP lacks the key subunits SRP9 and SRP14 that are involved in translational arrest or pausing in Eukaryotes [294] and thereby prevent premature synthesis of the IMP until the RNC-SRP complex has reached the ER. In *E. coli*, both SRP and FtsY are essential for growth suggesting their important role in inserting membrane proteins [160]. FtsY associates with the SRP-RNC whereupon the GTPase activity is activated and the RNC is transferred to the translocon [55,267]. FtsY binds to lipids but also directly to the translocon [7,8]. SRP-FtsY complex formation at the translocon prevents premature release of the RNC, and may ensure the efficient transfer of the RNC to the translocon as an elaborate rearrangement of the SRP-FtsY complex is required before the RNC can unload the substrate to initiate translocation [17,244]. A recent study suggests that the SRP targeting pathway is non-discriminatory and targets nascent cytosolic proteins to the translocon [29]. According to this model the nascent chains are selected at the translocon, and the signal-free nascent chains are rejected by the translocon for translocation initiation followed by release of the RNC into the cytoplasm.

The ribosome may not only be involved in polypeptide chain elongation and membrane protein insertion, it may also modulate and/or activate the translocon. The ribosomal exit tunnel is 100 Å long and about 10 – 20 Å wide [15,23,166,189]. Nascent proteins seem to already fold in the ribosomal tunnel [58,98,145,148,261]. Furthermore, the formation of hydrophobic folded TM domains in the ribosome exit

tunnel may promote ribosome-induced changes in the translocon itself [157]. Recently, it has been shown that the presence of a TM in the exit tunnel leads to the recruitment of the small tail-anchored membrane protein RAMP4 to the Sec61 translocon supporting the notion that there is communication between the ribosome and the translocon, preparing it for the arrival of a nascent TM segment [211].

Other proteins have been shown to interact with the ribosomal tunnel and thereby causing translational pausing [254]. SecM is a preprotein that is encoded by a gene that localizes upstream of the *secA* gene. A specific polypeptide sequence in SecM causes the translational arrest in the absence of an available translocon [178,180]. This specific motif binds to the ribosomal tunnel close to the polypeptide entry site, and the translational arrest results in unfolding of an mRNA hairpin that result in enhanced expression of the downstream encoded SecA protein. Thus SecM functions as a sensor to detect translocation defects in the cell [for review see [179]]. Recently, a similar kind of regulatory cascade was proposed for YidC-dependent membrane protein insertion in *B. subtilis* that contains two YidC homologs, SpoIIIJ and YqjG. A gene located upstream of *yqjG*, termed *mifM* seems to act as sensor of the SpoIIJ activity and that regulates YqjG expression. Decreased levels of SpoIIIJ results in an arrest in mifM translation causing the unfolding of a mRNA hairpin that blocks initiation of YqjG expression [43].

It has been suggested that in *E. coli* the ribosome interacts with YidC [114]. Thus, the SRP pathway would not only direct IMPs to the translocon but also to the YidC only pathway. How in such dual mechanism, specificity is maintained by the SRP pathway is currently unclear.

5. Mechanism of protein translocation

5.1. ATP and PMF-driven translocation

In *E. coli* the synthesis and translocation of the preproteins are uncoupled events [219], and preprotein translocation is mediated by the motor protein SecA. After a considerable portion of the preprotein has been synthesized at the ribosome, it can be bound by SecB that prevents it from stable folding and aggregation. SecB then targets the preprotein to the translocon where upon hydrolysis of ATP the preprotein is handed over to SecA [81]. Some preproteins are targeted to the translocon by SRP, but they still require SecA for translocation [237,250]. Both SecA, utilizing ATP, and the PMF are the driving forces of translocation across the membrane [62]. Not only is ATP essential for the initiation of translocation, it is also utilized throughout the translocation reaction as the energy source. After binding of ATP to SecA as the initial step, a hairpin loop of the signal sequence is inserted into the lateral gate of the translocon. While this step is solely dependent on ATP, it can be stimulated by the PMF. The PMF most likely plays a role in determining the correct orientation of the signal sequence within the channel [196,269], but alternatively, it may affect the conformation of the SecY protein and facilitate opening of the lateral gate region. The later suggestion is inspired by the observation that so-called PrlA mutations in SecY that cause a destabilization of the translocation pore, also cause translocation to be less dependent on the PMF [194]. Following translocation initiation, the hydrolysis of ATP leads to a dissociation of SecA from the preprotein and a weakening of the SecA-SecYEG binding affinity. SecA is then likely released from the translocon [236], but may rebind to the partially translocated preprotein thereby causing a

translocation of approximately 5 kDa of the preprotein through the channel [236,281]. Next binding of ATP to SecA causes a further translocation, likely of another 5 kDa, whereupon the ATP is hydrolyzed and a new catalytic cycle of SecA can be initiated. It is believed that multiple rounds of ATP binding and hydrolysis lead to the stepwise translocation of the preprotein. While the exact step-size has not been defined it has been demonstrated that duration of translocation is directly proportional to the length of the preprotein [257]. Various other factors such as the hydrophobicity of segments in the translocated protein may influence the kinetics of translocation [232]. Interestingly, once SecA has dissociated from the translocon, the PMF can continue to drive the translocation of the preprotein across the translocon [66,236,253,281]. While the PMF has been shown to participate and aid in translocation, it has also been shown to be involved in stimulating the release of ADP from SecA [248] as well as inducing conformational changes in SecA during protein translocation [184]. The PMF has also been implicated in channel opening [194,253]. Intermediate stages of translocation are reversible in the absence of ATP, SecA or the PMF and the preprotein shows hysteresis movements in the channel likely driven by folding at the cis and/or *trans* side of the membrane [66,236]. While tightly folded proteins can block translocation [9], it has been shown that SecA can display a sort of chaperone function by unfolding tightly folded proteins such as human I27 when presented at the C-terminal end of a preprotein [193].

5.2. Translocation models

Various models have been proposed for the SecA motor function, in particular the power-stroke and Brownian ratchet model [256]. With the power-stroke model,

the binding and hydrolysis of ATP results in the conformational change of the motor protein in such a manner that it imposes a mechanical insertion force on the translocating preprotein thereby pushing it through the protein conducting channel. It would seem that the two helix-finger shown to contact preproteins during translocation would fit with a power-stroke model [11,78], but currently there is no evidence that this region indeed moves in response to the nucleotide-bound state of SecA [311]. Moreover, it is difficult to envision how a movement of the two helix-finger can effect the translocation of about 25 amino acids per stroke. On the other hand, stepwise translocation might not depend on a large conformational change of the two-helix finger domain. A mechanical force on the translocating protein may also be affected by binding and release of SecA, which might involve a positioned interaction between the two-helix finger and the translocating preprotein. In order to effectively interact with an unfolded nascent chain, the two-helix finger needs to contact the amino acid side chains. Remarkably, the translocase can translocate long stretches of polyglycine of even up to 25 amino acids [192], and currently it is not clear how the two-helix finger would be able to interact with such polypeptide sequences.

The Brownian ratchet model suggests that SecA utilizes and directs the random Brownian motion of a unfolded translocating peptide [251]. Here, the retrograde movement of the preprotein in the channel is trapped by SecA in an energy dependent manner. This then leads to a directed translocation of the preprotein in a uni-directional fashion. Step-wise translocation involving distinct translocation intermediates [236,258,281] seems most consistent with a power-stroke mechanism, but an exact definition requires more accurate measurements of the translocation progression during the catalytic cycle.

Another model involves the dimeric structure of SecA [64]. In the antiparallel dimeric structure of SecA (**Fig. 3**), there is a central opening between the two SecA protomers. The piston-model proposes that this central pore aligns with the translocon and with ATP-binding traps the preprotein in a SecA-bound state. Utilizing a power-stroke mechanism, the preprotein would be pushed into the translocation channel [245]. This proposed mechanism was further adjusted and refined into the molecular peristalsis model [170] as discussed recently [65]. Briefly, this model requires the docking of a dimeric SecA onto a dimeric front-to-front oriented translocon (**Fig. 4**). Herein, translocation might occur through a consolidated channel formed by the two front-to-front translocons that would align with the central opening in the SecA antiparallel dimer. Conformational changes in SecA would not only affect preprotein trapping and translocation, but also cause the opening and closure of the translocon for translocation. The model proposes that there is an alternating opening and closure of the central SecA dimer channel that is synchronized with closure and opening of the protein conducting channel, thus leading to alternate trapping and release. Actual translocation in this model would be driven by Brownian motion, while the nucleotide binding and hydrolysis at SecA would be linked to channel opening and closure. A recent cross-linking study, however, suggest that the formation of a consolidated channel does not occur (F.P, unpublished results).

5.3. Proofreading by the translocon

An interesting set of mutants of the translocon are the so-called protein localization (*prl*) mutants. These mutations in the *sec* genes suppress the translocation defect of preproteins that have a defective targeting signal [25,26,57]. In SecY, most

of these mutations are localized at the inside of the pore of the translocon or are located on the plug domain (TM2a). Various explanations have been provided for the *prl* mutations in the SecY and SecE proteins. It has been suggested that these cause the loosening of the association of subunits of the SecYEG translocon [73]. While the *prl* mutants compensate for the defective signal sequence, it has also been suggested that these mutants either stabilize the open state or destabilize the closed state of the translocon. In *prl* mutants the interaction between SecYEG and SecA is stabilized [282]. In particular, there is a tighter binding of SecA in its ADP-bound state, resulting in suppression of the release of SecA from the translocation site. Consequently, translocation initiation is much more effective in the *prl* mutants as compared to the wild-type, and therefore translocation is more efficient in these mutants. In this respect, *prl* mutants are also seen as mutants with a defective proofreading allowing for more efficient translocation at the expense of specificity (defective signal sequences). This proofreading function seems related to the establishment of an actively primed state of SecA [182] that normally is dependent on the presence of a translocation competent preprotein with a functional signal sequence. In the *prl* mutants, this primed state is no longer dependent on the presence of a preprotein, and possibly corresponds to the 'pre-open' state of the translocon. Along these lines, these mutants are also less dependent on PMF for translocation [194] as discussed in a previous section. Overall, it appears that *prl* mutants mimick a SecA-SecYEG interaction where the SecA is in a constitutively active state [92,282]. In this respect, the signal sequence may indirectly activate SecA for ATP hydrolysis by inserting into the translocon, thereby promoting the open state of the channel which in turn may lead to an activation of the SecA ATPase.

6. Mechanism of membrane protein integration

6.1. Signals for membrane protein insertion

For membrane proteins, integral signals in their TMs are read and decoded by the translocon or alternatively by YidC. Typically, TMs of membrane proteins in the inner membrane consist of hydrophobic α-helices that contain around 20 to 27 residues. These helices are inserted perpendicular to the membrane (**Fig. 2**A) and often are found tilted in the membrane. While most proteins destined for secretion contain a cleavable signal sequence, very few integral membrane proteins contain a cleavable signal peptide. Those that do contain a cleavable signal peptide contain a second hydrophobic sequence also known as a stop-transfer sequence [291,293]. Deletion of this stop-transfer sequence can lead to a conversion of the membrane protein into a secreted protein [3,47]. The reverse is also possible where a secreted protein is converted into a membrane protein by the addition of a stop-transfer sequence [48]. For a review of typical topologies of membrane proteins and a summary of their requirements for insertion see [301] and [79]. For multispanning membrane proteins, the TM domains need to fold and pack to form a functional protein. Various factors have been proposed to affect the folding of large multispanning proteins; in particular YidC has been suggested as folding chaperone. For instance, YidC has been shown to be essential for the correct folding of the 12 TM spanning LacY [175]. As YidC has been shown to contact TMs that exit the translocon [113] it has been proposed that it can act as a chaperone for membrane protein folding. However, with LacY, SecY mutations have been identified that affect the correct folding of the protein [247], suggesting that SecY together with YidC

synergistically affect the folding of membrane proteins. Once inserted, membrane proteins may assemble into multimeric protein complexes. This process requires that subunits are present in the correct stoichiometry and assembly likely occurs in a specific order. Moreover, for complexes like the F_1F_0 ATPase, various subunits may utilize different insertion routes. For instance, subunit c of the F_0-sector inserts into the membrane via YidC [274] while subunit a requires both YidC and SecYEG [141,304]. Furthermore, subunit c needs to assemble first into the c-ring before it associates with the ab_2 sub-complex. It could well be that the assembly process also depends on YidC, a concept which is further re-enforced by observations that the entire F_1F_0 ATPase co-purifies with the *B. subtilis* YidC homologs [228]. The control and regulation of the assembly of these large energy transducing complexes remain a subject of further study.

The insertion and final topology of membrane proteins is also influenced by the PMF, in particular the transmembrane potential, $\Delta\psi$. In the early 1980's it was shown that the membrane insertion of the procoat protein into the *E. coli* cytoplasmic membrane strictly depends on the $\Delta\psi$ [46]. While binding to the membrane occurs in the absence of a $\Delta\psi$ [87], translocation of the periplasmic loop that connects the two TM-like domains of procoat is dependent on this force. Mutagenesis of the negatively charged amino acid residues in this loop into neutral or positively amino acids results in $\Delta\psi$-independent membrane insertion [37,152,239]. Since YidC is responsible for the insertion of M13 procoat [41,151,229], it remains a major question as to whether $\Delta\psi$ acts by electrophoresis or whether it functions via YidC. In this respect, Pf3 variants that exhibit a reduced $\Delta\psi$ dependence still require YidC for membrane insertion. Moreover, other membrane proteins also depend on the $\Delta\psi$ for insertion by as yet unknown mechanisms.

A major topology-determining factor for membrane protein insertion is the "positive inside rule" of von Heijne and co-workers. This rule states that the positively charged amino acid residues flanking the transmembrane domains are topology determining and remain in the cytosol during biogenesis [290,292]. However, introduction of negatively charged amino acids at the cytosolic face of the membrane of a membrane protein can negatively influence insertion and topology [155]. Here, the negatively charged amino acid residues seem to respond to the presence of $\Delta\psi$. Whereas $\Delta\psi$ supports the translocation of negatively charged amino acid residues and inhibits the translocation of positively charged amino acid residues [132]. Recently, Seppälä and co-workers investigated the insertion of multispanning membrane proteins with regard to the role that positive charges play in the topology of this class of proteins [242]. Surprisingly, they found that the topology of EmrE, a topological sensitive protein, comprising of four or five transmembrane helices could be controlled by the placement of a single positively charged amino acid residue at various locations within the protein. While EmrE is sensitive to its orientation, this study highlights the effect that a single positive charge can have on the overall topology of a protein, even when this charge is found on the very C-terminus of the protein. To understand the mechanism how this is accomplished a detailed investigation of the insertion and release of TMs from multispanning membrane proteins via the translocon is required.

6.2. Thermodynamic mechanism of translocon-dependent partitioning of transmembrane domains

How does the translocon identify and select TMs for insertion? Membrane proteins are highly prone to aggregation when released into the cytosol by the ribosome. Therefore, the ribosome and the translocon work together with the SRP targeting pathway to insert membrane proteins into the lipid bilayer. When a TM that is exposed from the ribosome associates with the translocon, it needs to be recognized such that it can be released laterally in the membrane. Furthermore, the TM segments of multispanning membrane proteins need to assemble into a functionally folded protein. Newly synthesized membrane proteins rapidly equilibrate with the lipid bilayer after their insertion [156,297]. The thermodynamics of this process was recently studied utilizing model TMs [107]. Insertion is determined by the average hydrophobicity of TM segments quantitatively described by the Gibbs free energy of insertion (ΔG_{app}) [107]. This suggests that insertion involves partitioning between a polar and apolar environment, possibly involving the lateral diffusion of the inserting TM segment into the lipid bilayer [102]. Alternatively, the hydrophobicity of the TM segment controls the gating of the translocation channel [308]. To discriminate between above possibilities it has to be determined whether TM domains first insert into the aqueous translocation pore before their lateral release into the lipid bilayer. Importantly, insertion is also kinetically controlled [263], and moderately hydrophobic polypeptide domains may insert into the membrane when translocation (or translation) is slow [72]. In another study the effect of the position of a specific amino acid in the TM were investigated [107]. When the polar amino acid arginine is positioned closer to the centre of the TM segment, the greater the energy cost to insert

the TM. This suggests that protein-lipid interactions are crucial for the translocon to recognize the inserting TM segment. Interestingly, a significant number (25%) of TMs in multispanning membrane proteins exhibit a predicted ΔG_{app} greater than 0 [108], suggesting that elements other than the translocon or mere water-lipid partitioning contribute to their insertion. Insertion of TM helices also depends on the presence and composition of the neighboring TM helices for proper insertion. Membrane insertion via YidC seems to follow similar principles [302].

A study on the insertion of aquaporin which consists of 4 TM segments, via the Sec61 translocon [227] has revealed that the TM segments leave the translocon in the same order as they are released from the ribosome. However, it was also observed that TM segments once released by the translocon can return at a later stage when other TM segments enter the lipid bilayer. This suggests a mechanism by which TM segments help each other in the lateral release into the lipid bilayer, possibly by promoting proper protein folding. Von Heijne and coworkers studied the phenomenon of marginally hydrophobic TMs [101]. They screened 16 TM domains with marginal hydrophobicity for their insertion as individual TMs or in the context of flanking TMs and loops. Most of the marginally hydrophobic TM domains appeared insufficient to stably insert on their own, and required flanking hydrophobic TMs for insertion. A study on the insertion of short TMs [118] revealed that the efficiency of insertion of TMs via the translocon is determined by the length of the TM, its amino acid composition and the positional arrangement of amino acids within the TM domain. However, the variety of structural elements found in membrane proteins that might influence the insertion of TMs is far from understood as insertion seems not only to depend on the physicochemical properties of the TM domains but also on the activity of the translocon. In this respect, mutations have been described in SecY (Sec61α)

that affect the final topology of the inserting membrane protein. This suggests an active role of the translocon in membrane protein insertion.

Finally, membrane insertion being tightly linked to polypeptide chain elongation at the ribosome must synchronize with the SecA motor function. When large polar domains emerge from the ribosome tunnel that need to be translocated, SecA needs to bind to the nascent chain that is exposed to the cytosol and dissociate the ribosome from its SecYEG bound state. Interestingly, under in vitro conditions where post-translational membrane insertion was enforced, SecA was released from the polypeptide chain once the SecA encountered a hydrophobic transmembrane segment [109]. Future studies should address the exact mechanism of the interplay between the ribosome and SecA, and how their binding to SecYEG is coordinated.

7. The role of lipids in translocation and membrane protein insertion

Typically, the inner membrane of *E. coli* consist of roughly 75% PE (phosphatidylethanolamine), 20% PG (phosphatidylglycerol) and around 5% of cardiolipin [218]. Interestingly, PE has the propensity to form inverted non-bilayer structures when in isolation. Therefore, being the most abundant lipid in the membrane it creates a certain level of curvature stress, a condition which has been proposed to play a significant role in protein function [54]. *In vivo*, a strain lacking PE is viable only in the presence of a high concentration of divalent cations such as Mg^{2+} or Ca^{2+}. In this strain, the lack of PE is compensated by increased levels of PG and cardiolipin and it has been argued that the presence of divalent cations enforces the type II hexagonal phase structure of cardiolipin consistent with a strong requirement for non-bilayer lipids. Indeed, membrane vesicles from the same strain show a severe

defect in protein translocation in the absence of divalent cations, and this could be rescued by the re-introduction of PE into the vesicles [224]. A strain in which the phosphatidylglycerol synthetase gene was depleted is devoid of PG and cardiolipin shows a lethal phenotype, but accumulates increased levels of the negatively charged phospholipids phosphatidylserine and phosphatidic acid. This phenomenon can be attributed to a loss of the anionic phospholipid PG as the inactivation of the cardiolipin synthetase genes has no effect on growth. Remarkably, a recent study suggested that cardiolipin stimulates the formation of the SecYEG dimer [89] despite the fact that cardiolipin is non-essential for protein translocation. Both in vivo and in vitro studies demonstrate that in the absence of PG, protein translocation is severely impaired [56]. By reintroducing either PG or any other anionic phospholipid, this deleterious effect could be rescued, indicating that the negative charge on the polar head group of the phospholipid is essential for protein transport [153]. Furthermore, anionic lipids have also been found to be essential for the activity of SecA by increasing the affinity of binding to SecY and stimulating the hydrolysis of ATP [100,105,159]. Reconstitution studies with the purified SecYEG complex confirmed the requirement for non-bilayer lipids as well as the need for anionic phospholipids for protein translocation [273]. Interestingly, these studies suggest a bulk requirement for such lipids as the optimal protein translocation activity with the *E. coli* and *B. subtilis* SecYEG complexes is observed with a synthetic mixture of phospholipids that corresponds to the polar head group composition of the respective species. The role of lipids in the SRP targeting pathway has also been investigated. Lipids are important for the release of SRP molecule from the nascent chain [241,267]. Moreover, FtsY interacts specifically and peripherally with the headgroups of PE (or PC) via its AN domain [168]. Recently, it has been shown that lipids stimulate the GTPase activity of

FtsY [14]. Analysis of FtsY mutants indicated that the N-domain of FtsY contains an amphipathic lipid-binding domain that is essential for its function in vivo [205]. Leader peptidase requires anionic membrane lipids for its insertion [284] and to obtain the correct topology [283]. This phenomenon has been attributed to an anionic lipid requirement for SecA activity needed to translocate the polar catalytic domain of leader peptidase across the membrane. Obviously, many catalytic and structural aspects of membrane proteins are affected by lipids in the bilayer. For a recent review on the role of lipids in determining membrane protein topogenesis see [27] and [61]. For a review specifically on the interaction of lipids with membrane proteins, with references to the structure see [116] and [117]. The proposed model of a lateral gate opening of the translocon and the possible mechanism of insertion of TM domains suggests that lipids might play a more direct role in the insertion process. This possible function, however, needs to be studied in further detail.

8. Concluding Remarks

Here, we have given an overview of the recent insights in the process of protein translocation and membrane protein biogenesis in Bacteria. Although in recent years significant insights have been obtained in the structural and functional roles of the various components of the translocase, major questions still remain unresolved as for instance the mechanism by which TM domains exit ribosomes and the translocon. Obviously, any proposed mechanism will need to prevent the uncontrolled leakage of ions through an aqueous pore, while a laterally opened channel should not compromise the water-filled integrity of the translocon. Despite the multitude of approaches employed, the debate concerning the oligomeric state of the translocon

has still not been resolved. This is mostly due to fact that in many of the experimental conditions used for the analysis of the oligomeric state of the translocon, the functionality of the translocon is not guaranteed. Another concept emanating from this review is the amazing complexity observed for the insertion of membrane proteins into the lipid bilayer. In particular, the question whether the ribosome fulfils a role in early recognition and in controlling the opening and closure of the translocon remains to be investigated. YidC has so far resisted structural elucidation, and this has hampered our insights in the molecular basis of the YidC function, which ranges from membrane protein insertion, assembly and folding. While there is a high level of conservation between the essential components of the translocase in Eukaryotes, Bacteria and Archaea, there are significant differences between these systems that warrant independent investigations of catalytic mechanism of protein translocation and membrane protein insertion. For instance, Archaea lack a clear homolog of the SecA translocation ATPase suggesting that translocation in these organisms is either co-translationally or involves some novel motor protein(s). Mechanistic studies on the translocase will benefit from investigations at the single molecule level to reveal intimate features of the translocation reaction and the coupling between ATP and translocation progress.

Acknowledgments

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Scope of this thesis

With the recent availability of the X-ray structure of the SecYEβ translocon from *Methanocaldooccus jannasschii* investigation into the structure-function relationship of the translocon is now possible. In addition, a new membrane protein insertion pathway has been discovered based on the activity of YidC sofar restricted to small membrane proteins only. On the other hand, membrane subunits from respiratory chain complexes such as cytochrome oxidase seem to dependent on a concerted activity of the translocon and YidC for their insertion. The scope of this thesis is to investigate the mechanism of co-translational insertion of membrane proteins in *E. coli* and to understand how this is linked to conformational changes in the translocon. The experimental approach involved coupled *in vitro* transcription-translation-insertion assays utilizing both membrane vesicles as well as proteoliposomes reconstituted with purified translocon components. In addition, site-directed cysteine mutagenesis was used to investigate specific aspects of the structure-function relationships inspired by the structure of the SecYEβ translocon. In **Chapter 2**, we investigated the minimal requirements for the membrane insertion of CyoA, the quinol binding subunit of the cytochrome o oxidase. A new insertion pathway was identified showing CyoA requires both the translocon, SecA as well as YidC for its correct insertion into the inner membrane. **Chapter 3** examines the role of the proposed lateral gate between TM2b and TM7 of SecY in both protein translocation and membrane protein insertion. Here, site-directed mutagenesis was employed to introduce cysteines in the lateral gate and to generate stable crosslinks that trap the lateral gate in a closed state. In addition, by the use of crosslinkers with various spacer lengths flexibility was re-introduced into the lateral gate. The data shows that protein

translocation depends on flexibility in the lateral gate region and suggests that the activation of the SecA ATPase is allosterically linked to lateral gate opening. The **Appendix to Chapter 3** describes the role of the lateral gate in membrane protein insertion. Surprisingly, disulfide-bonding induced closure of the lateral gate had no effect on the translocon dependent membrane insertion of the mannitol permease, MtlA, a large 6 TM spanning protein, that does not require SecA for membrane insertion. These data suggest that lateral gate opening is not needed for the membrane insertion of TM segments. **Chapter 4** describes a directed cysteine mutagenesis approach to investigate the functionality of the suggested front-to-front model of the dimeric translocon in both protein translocation and membrane protein insertion. Crosslinking of the front-to-front arrangement that excluded the possibility of the translocon to form a consolidated pore had no effect on the functionality of the translocase. This suggested that a consolidated pore is not formed during translocation. Finally, **Chapter 5** summarizes the results of this work and provides an outlook for future studies.

Chapter 2

Subunit a of cytochrome *o* oxidase requires both YidC and SecYEG for membrane insertion

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Summary

The *Escherichia coli* YidC protein belongs to the Oxa1 family of membrane proteins that facilitate the insertion of membrane proteins. Depletion of YidC in *E. coli* leads to a specific defect in the functional assembly of major energy transducing complexes such as the F_1F_0 ATPase and cytochrome bo_3 oxidase. Here, we report on the *in vitro* reconstitution of the membrane insertion of the CyoA subunit of cytochrome bo_3 oxidase. Efficient insertion of *in vitro* synthesized pre-CyoA into proteoliposomes requires YidC, SecYEG, and SecA and occurs independently of the proton motive force. These data demonstrate that pre-CyoA is a substrate of a novel pathway that involves both SecYEG and YidC.

Introduction

Approximately 20% of the *Escherichia coli* proteome concerns inner membrane proteins [150]. Most of these proteins insert into the membrane via the Sec translocase (for review, see Ref. [45]. Recently, YidC has been identified as a novel membrane protein that facilitates insertion of a subset of membrane proteins on its own [229,243,274]. YidC also associates with SecYEG [229], where it contacts transmembrane (TM) insertion segments of newly synthesized membrane proteins [111,112,240]. YidC is homologous to Oxa1 in mitochondria and Alb3 in chloroplasts [229]. The latter two proteins act as membrane protein insertases and play an important role in the membrane insertion of subunits from major energy transducing complexes (for review, see Refs. [277] and [305]). In analogy, in *E. coli* the functional assembly of the F_1F_0 ATPase and cytochrome bo_3 quinol oxidase is shown to be dependent on YidC [278], and YidC is also implicated in lipoprotein translocation [85]. We have recently demonstrated that membrane insertion and assembly of the F_0c subunit of the F_1F_0 ATPase solely depend on YidC [274]. CyoA is the quinol binding subunit of the cytochrome bo_3 quinol oxidase complex [2]. Unlike F_0c, CyoA is a polytopic membrane protein with a lipoprotein signal sequence and a large periplasmic domain (**Fig. 1**A). Here we report on the minimal requirements for insertion of pre-CyoA into the *E. coli* membrane using an *in vitro* approach. The data demonstrate that pre-CyoA is a substrate of a novel pathway that requires both the Sec translocase and YidC.

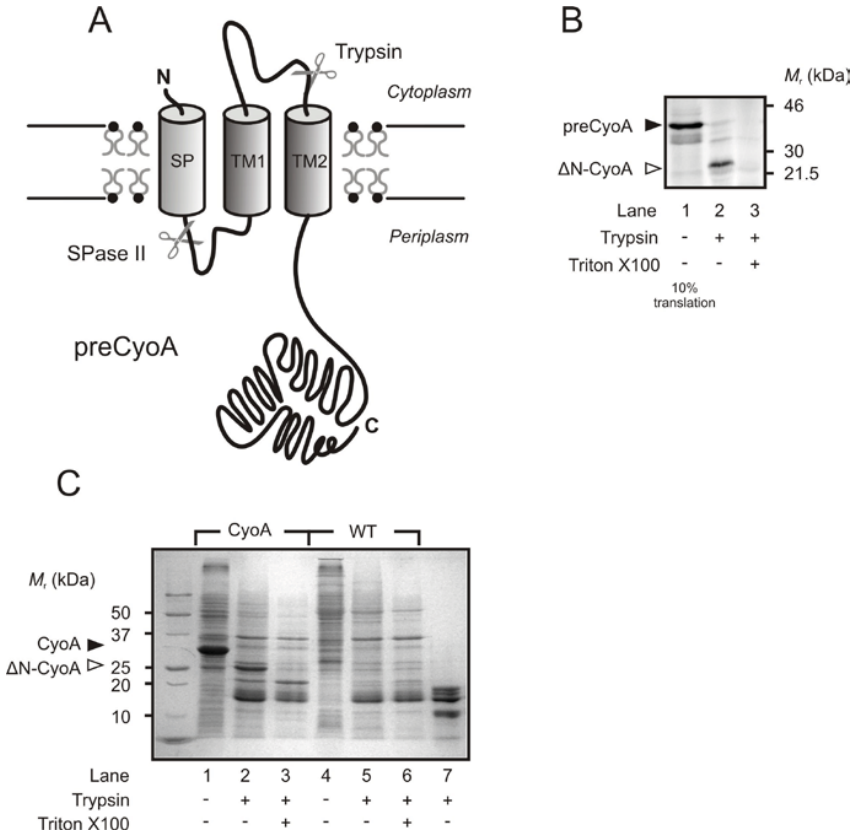

Fig. 1 *In vitro* insertion of preCyoA into *E. coli* inner membrane vesicles. (**A**) Schematic representation of the membrane topology of preCyoA before removal of the signal sequence by signal peptidase II (SPase II). The trypsin cleavage site in the cytoplasmic loop is indicated by an arrow. (**B**) Coupled *in vitro* transcription/translation of preCyoA in the presence of 25 µg of SF100 IMVs containing high levels of SecYEG complex (lane 1, 10 % of the total translation). Samples were treated with trypsin in the absence (lane 2) or presence (lane 3) of 1 % Triton X-100. Full length preCyoA and the trypsin protected fragment (ΔN-CyoA) are indicated. (**C**) Coomassie stained SDS-PAGE gel of IMVs from *E. coli* SF100 with (lanes 1-3) and without (lanes 4-6) overexpressed CyoA. Samples were treated with trypsin in the absence (lanes 2 and 5) or presence of 1% Triton X-100 (lanes 3 and 6). As a reference, trypsin was loaded in lane 7.

Results

Co-translational insertion of pre-CyoA into inverted E. coli inner membrane vesicles

Subunit II (CyoA) of cytochrome bo_3 ubiquinol oxidase (315 residues) from *E. coli* is synthesized as a precursor with an N-terminal signal sequence (pre-CyoA) that upon lipid modification of the mature N terminus is cleaved by signal peptidase II [162]. Mature CyoA with a mass of 32 kDa is composed of two domains, an N-

terminal membrane region with two TM domains and a large periplasmic C-terminal domain [2] (**Fig. 1**A). To study its membrane insertion, pre-CyoA was synthesized *in vitro* using an *E. coli* S135 lysate and [^{35}S]methionine. *In vitro* synthesis of CyoA results in the formation of a 35-kDa protein visualized on SDS-PAGE (**Fig. 1**B, *lane 1*). When the *in vitro* transcription/translation reaction was performed in the presence of SecYEG-overexpressed IMVs, trypsin treatment of pre-CyoA resulted in the formation of a 25-kDa protease-protected fragment (**Fig. 1**B, *lane 2*). Solubilization of IMVs with Triton X-100 resulted in complete degradation of pre-CyoA (**Fig. 1**B, *lane 3*). In its correct topology, the large periplasmic domain of CyoA is translocated into the vesicle lumen and thus becomes protected from externally added trypsin. The cytoplasmic loop connecting TM1 and TM2, however, will be accessible to trypsin. Based on the available crystal structure of CyoA [1], this cytoplasmic loop contains four possible trypsin cleavage sites (at amino acid positions 70, 74, 77, and 87). Trypsin cleavage at one or all of these sites will result in the removal of the signal sequence and part of N-terminal region of the mature CyoA yielding a ~25-kDa fragment (ΔN-CyoA). Correspondingly, trypsin treatment of endogenous CyoA in inside-out IMVs yielded a 25-kDa protease-protected fragment that degraded upon solubilization of the membrane vesicles with Triton X-100 (**Fig. 1**C). We therefore conclude that the *in vitro* observed 25-kDa trypsin-protected fragment in the presence of IMVs represents correctly membrane-inserted CyoA.

To examine the insertion mechanism of pre-CyoA, IMVs with high levels of SecYEG were used as described previously [271]. Levels of overexpression for SecYEG were calculated to be at least 10-fold that of wild-type levels of SecYEG (see also **Fig. 7**). Although wild-type IMVs showed only a low level of inserted CyoA

(**Fig. 2**, *lane 2*), overproduction of SecYEG (SecYEG$^+$) enhanced membrane insertion more than 5-fold (*lane 5*). This correlates well with the observed 5–6-fold stimulation of proOmpA translocation into IMVs upon SecYEG overexpression (data not shown) [272] and shows that insertion of pre-CyoA is a SecYEG-mediated process. The low level of membrane insertion with wild-type IMVs has been observed more often with *in vitro* systems [276] and likely results from a general inefficiency of *in vitro* translation/translocation reactions for inner membrane proteins and competing reactions such as aggregation. Other missing factors may contribute to the efficiency of membrane insertion, such as an intact lipid modification pathway needed to modify the mature N terminus of pre-CyoA prior to its processing by the lipoprotein peptidase. Finally, co-factor assembly and CyoB maturation may contribute to the overall efficiency of stably inserted CyoA.

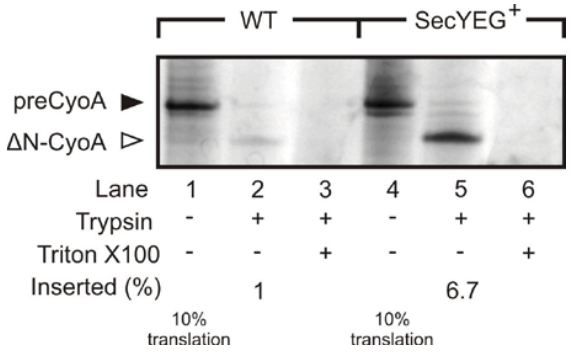

Fig. 2 Membrane insertion of preCyoA is facilitated by SecYEG. PreCyoA was synthesized in the presence of 25 µg wild-type or SecYEG$^+$ IMVs. After 40 min at 37°C, samples were treated with trypsin without (lanes 2 and 5) or with 1 % Triton X-100 (lanes 3 and 6) for 30 min on ice and analysed by SDS-PAGE and autoradiography. Lanes 1 and 4 represent 10 % of the total translation.

In the *in vitro* assays, pre-CyoA was synthesized in the presence of IMVs (co-translational insertion). To investigate whether CyoA also inserts post-translationally, pre-CyoA was first synthesized in the absence of IMVs. Next, protein synthesis was blocked by chloroamphenicol, and SecYEG$^+$ IMVs were added to allow insertion

(**Fig. 3**, lanes 4-6). Although efficient insertion of pre-CyoA was observed under co-translational conditions (**Fig. 3,** *lane 5*), no pre-CyoA insertion could be detected under post-translationally conditions (*lane 2*). These data demonstrate that membrane insertion of pre-CyoA occurs co-translationally.

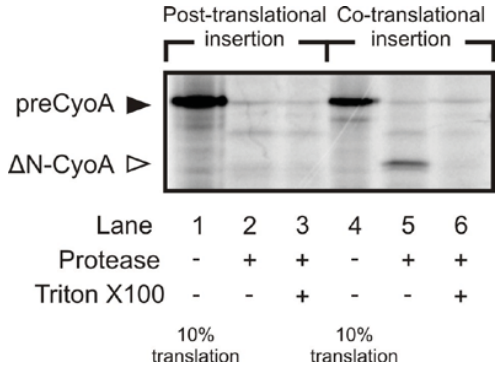

Fig. 3 PreCyoA inserts co-translationally into IMVs. Co-translational *in vitro* insertion of preCyoA (lanes 4-6) was performed using a coupled transcription/translation reaction in the presence of 25 µg SecYEG$^+$ IMVs. Lane 4 represents 10 % of the total translation. After 40 min incubation at 37°C, samples were treated with trypsin in the absence (lane 5) or presence of 1 % Triton X-100 (lane 6). Post-translational insertion of preCyoA (lanes 1-3) was done as above but in the absence of IMVs (lane 1 represents 10% of the total translation). Translation was terminated by addition of 25 µg/ml chloramphenicol, and subsequently 25 µg SecYEG$^+$ IMVs were added and the incubation was continued for 40 min at 37°C.

The proton motive force is not required for membrane insertion of CyoA

The proton motive force (PMF) has been shown to play a pivotal role in the insertion of some membrane proteins such as M13 procoat [37] and FtsQ [276]. Previously, we have shown that YidC depletion from cells results in a reduced capacity of cells to generate a PMF [278]. The observed assembly defect of CyoA in YidC-depleted cells could therefore relate to a PMF requirement of the insertion reaction. Therefore, the role of the PMF in pre-CyoA insertion was examined *in vitro*. Insertion of pre-CyoA into wild-type and SecYEG$^+$ IMVs was only marginally affected by the ionophores nigericin and valinomycin that collapse the entire PMF

(**Fig 4**A). In contrast, ionophore addition completely blocked membrane insertion of the control membrane protein FtsQ (**Fig. 4**B) [276]. These results demonstrate that membrane insertion of pre-CyoA occurs independently of the PMF.

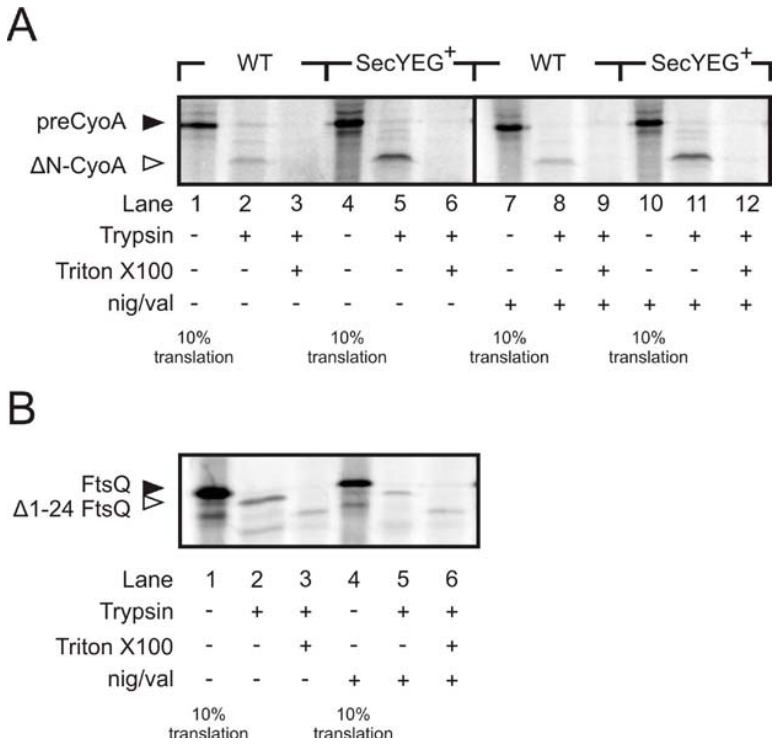

Fig. 4 Insertion of preCyoA does not require a PMF. (**A**) Insertion assays with wild-type and SecYEG$^+$ IMVs were performed as in the legend to Fig. 2 in the absence (lanes 1-6) and presence (lanes 7-12) of 3 µM nigericin/valinomycin (Nig/Val) to dissipate the PMF. (**B**) A coupled transcription/translation of FtsQ was performed in the presence of 25 µg of wild-type IMVs in the absence (lanes 1-3) and presence (lanes 4-6) 3 µM Nig/Val to dissipate the PMF.

Membrane insertion of CyoA requires both SecYEG and YidC

To investigate the minimal requirements for insertion of pre-CyoA, proteoliposomes were used that contained purified YidC, SecYEG, or both YidC and SecYEG. Herein, a molecular YidC/SecY ratio of 3 was used as described previously [275]. No insertion was observed when pre-CyoA was synthesized in the presence of empty liposomes (**Fig 5**A, *lane 2*) or proteoliposomes reconstituted with YidC only (*lane 11*). A low level of insertion was observed with proteoliposomes containing

purified SecYEG (**Fig 5**, *lane 5*), but co-reconstitution of YidC with SecYEG resulted in a drastic increase in the membrane insertion efficiency of pre-CyoA (*lane 8*). The increased level of pre-CyoA insertion was not because of differences in SecYEG reconstitution as the liposomes equally effective translocated the precursor protein proOmpA (**Fig 5**B, *lanes 3* and *4*). A further increase in the amount of YidC in the proteoliposomes only marginally improved the insertion (data not shown). Taken together, the above results indicate that both SecYEG and YidC are required for efficient membrane insertion of pre-CyoA.

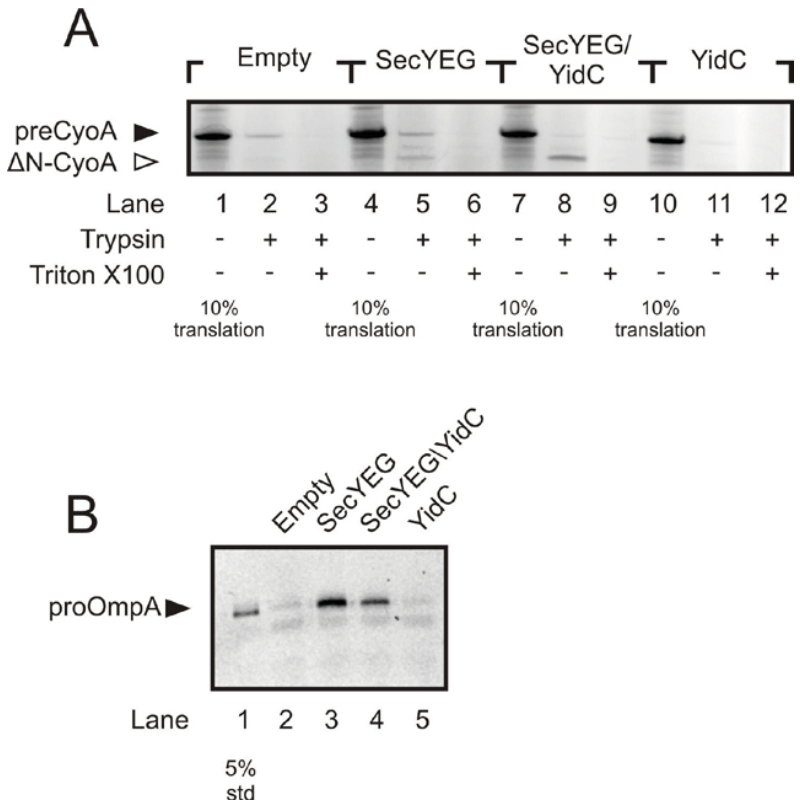

Fig. 5 Efficient insertion of CyoA into proteoliposomes requires both SecYEG and YidC. Liposomes were reconstituted with purified SecYEG (20 µg) and/or YidC (60 µg) as described in the methods section. (**A**) PreCyoA was synthesized in the presence of proteoliposomes containing SecYEG (lanes 4-6), SecYEG and YidC (lanes 7-9), YidC (lanes 10-12) or liposomes (lanes 1-3). Samples were treated with trypsin in the absence (lanes 2, 5, 8 and 11) or presence (lanes 3, 6, 9 and 12) of 1% Triton X-100. (**B**) Fluorescein-labelled proOmpA was translocated into liposomes (lane 2), or proteoliposomes containing SecYEG (lane 3), SecYEG and YidC (lane 4) or YidC alone (lane 5).

Membrane insertion of pre-CyoA is dependent on SecA

Membrane proteins with large periplasmic domains such as FtsQ [265,276], AcrB [217], and YidC [138] have been shown to require SecA for membrane insertion. As CyoA contains a large periplasmic domain (**Fig 1**A), we next determined the SecA dependence of the insertion reaction. Pre-CyoA was synthesized in the presence of SecYEG/YidC proteoliposomes in a SecA-immunodepleted *E. coli* lysate. Although the lysate supported synthesis of pre-CyoA, no insertion could be observed (**Fig. 6**, *lanes 4-6*). When the lysate was supplemented with purified SecA, pre-CyoA insertion was restored (**Fig. 6**, *lanes 7-9*). This demonstrates a catalytic requirement for SecA.

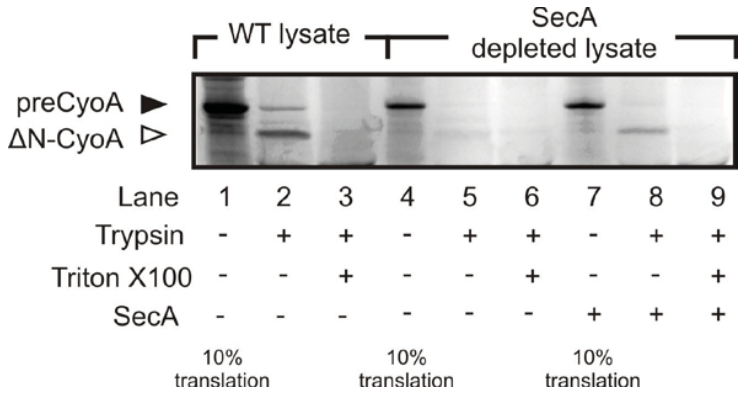

Fig. 6 SecA is required for membrane insertion of preCyoA. preCyoA was synthesized in an *E. coli* wild-type lysate (lanes 1-3) and in an SecA immunodepleted lysate without (lanes 4-6) or with 0.5 µg purified SecA (lanes 7-9). Insertion assays were performed with SecYEG/YidC proteoliposomes.

Mutations in SecY have been described that differently affect protein translocation and membrane protein insertion [13]. SecY39 (R357E mutation in the C5 cytoplasmic loop of SecY) is blocked in protein translocation [13,246] and exhibits a functional defect in the SecA/SecY interaction [172]. This mutant is also defective in the insertion of some signal recognition particle-dependent membrane

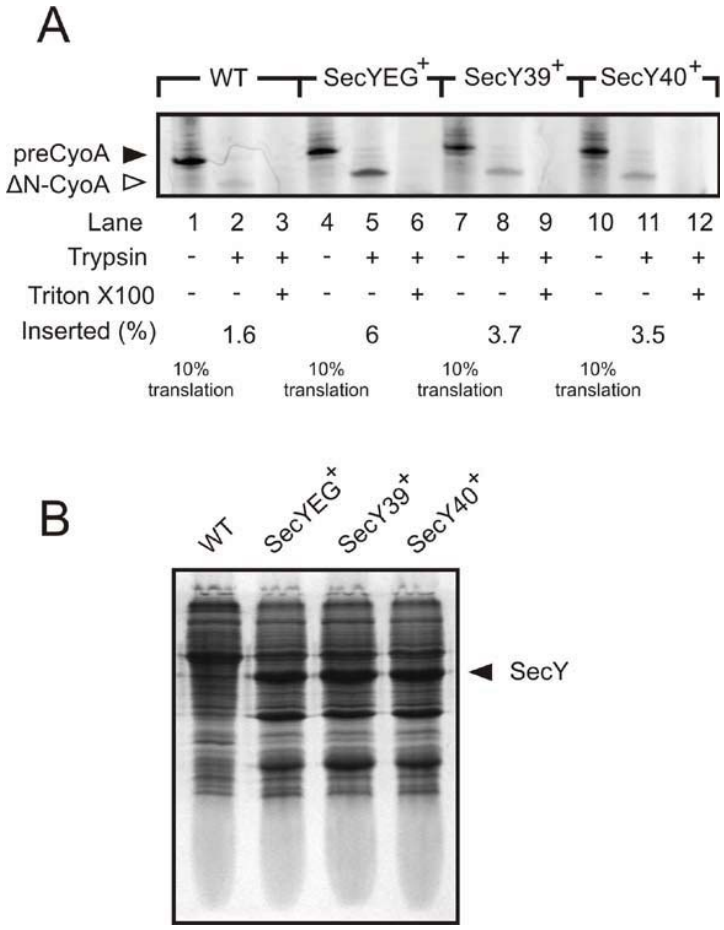

Fig. 7 SecY mutations interfere with insertion of preCyoA into IMVs. (**A**) PreCyoA was synthesized in the presence of wild-type (lanes 1-3), SecYEG$^+$ (lanes 4-6), SecY(R357E)EG$^+$ (lanes 7-9) or SecY(A363S)EG$^+$ (lanes 10-12) IMVs. Samples were treated with trypsin in the absence (lanes 2, 5, 8 and 11) or presence (lanes 3, 6, 9 and 12) of 1% Triton X-100. (**B**) Coomassie brilliant blue stained SDS-PAGE of equal amount of wild-type, SecYEG$^+$, SecY(R357E)EG (SecY39$^+$) and SecY(A363S)EG (SecY40$^+$) IMVs With the SecY level of SecYEG$^+$ IMVs set at 100%, SecY39$^+$ and SecY40$^+$ IMVs contained a SecY level of 107% and 103%, respectively.

proteins [139,172]. SecY40 (A363S) is defective in signal recognition particle-dependent membrane protein insertion but supports normal protein translocation [8,246]. As pre-CyoA is a protein that contains both TM domains and a large periplasmic domain, we determined the effect of the SecY mutations on the membrane integration of pre-CyoA. IMVs were isolated from cells overproducing SecY(R357E)EG and SecY(A363S)EG and analyzed for pre-CyoA insertion. Coomassie-stained SDS-PAGE analysis showed that the SecY mutant proteins were

overproduced to the same level as wild-type SecY (**Fig 7**B). Both SecY(R357E)EG (**Fig. 7**A, *lanes 7-9*) and SecY(A363S)EG (*lanes 10-12*) IMVs showed a severe defect in the membrane integration of pre-CyoA as compared with $SecYEG^+$ IMVs (*lanes 4-6*). The residual level of insertion above that of IMVs containing chromosomal levels of SecYEG (*lanes 1-3*) is in line with previous observations that these mutants are not completely defective [13]. Taken together, these data indicate that pre-CyoA is a substrate of a novel route that involves both the Sec translocase and YidC.

Discussion

Recently, we have shown that in *E. coli* the functional assembly of major energy-transducing complexes such as the H^+-translocating F_1F_0 ATPase and cytochrome bo_3 oxidase is strongly affected by the depletion of YidC [278]. *In vitro* experiments demonstrate that the membrane insertion of F_0c is solely mediated by YidC [274], thus establishing a novel route for membrane insertion of authentic *E. coli* membrane proteins, which involves only YidC. YidC is also required for membrane insertion of foreign small phage proteins such as M13 and Pf3 [229,243,274] that apparently usurp the YidC pathway for their insertion. YidC also interacts with the SecYEG complex, and cross-linking approaches have shown that it contacts the TMs of newly inserted membrane proteins [111,112,229,240]. The role of YidC in the membrane insertion of these Sec-dependent membrane proteins is less understood as no strict requirement for YidC is demonstrated for their functional assembly [229,276]. We now show that pre-CyoA, the precursor of subunit a of the cytochrome bo_3 quinol oxidase complex, utilizes both the Sec translocase and YidC for its insertion. We used an *in vitro* assay, which employed proteoliposomes with a

defined protein composition, to reveal the minimal requirements for membrane insertion of pre-CyoA. For the first time, our data demonstrate a catalytic requirement for YidC by a membrane protein that inserts into the membrane in a Sec-dependent manner. This study explains why depletion of YidC in cells results in a loss of functional cytochrome *o* oxidase complex. Pre-CyoA insertion also requires SecA for its assembly, which most likely relates to the translocation of the large periplasmic domain of CyoA as expected for membrane proteins with periplasmic domains larger than 60 amino acids [6].

Pre-CyoA membrane insertion presumably occurs in the following manner. First, the signal sequence and the first transmembrane segment insert into the SecYEG channel as a helical hairpin. This state may resemble the recent cryo-electron microscopy reconstruction of a ribosome-SecYEG complex in which the N-terminal TM domain of FtsQ was inserted as a hairpin structure [171]. This process is likely followed by the lipid modification of the cysteine position of the mature N terminus of CyoA and then by the removal of the signal sequence by signal peptidase II. There are processes that are not monitored in the *in vitro* system as described in this study. During the lipid modification, TM2 of CyoA (**Fig. 1**A) must insert into the membrane, whereupon the large periplasmic domain of CyoA needs to be translocated across the membrane. TM2 likely loops into the SecYEG pore together with the N-terminal region of the periplasmic domain of CyoA. The translocation of the periplasmic domain likely involves SecA as this reflects a true translocation reaction. YidC may be involved in various stages of the insertion reaction. It may facilitate clearance of the SecYEG pore and promote transfer of the hairpin of the signal sequence and TM1 into the lipid phase. Alternatively, YidC may be involved in the insertion of TM2 that needs to loop into the translocation pore. The latter process

resembles the insertion mechanisms of F_oc and M13 in which YidC may facilitate looping in of a single or of both TM domains of these small membrane proteins. Future experiments should reveal how YidC facilitates membrane insertion of the various regions of CyoA.

CyoA is the quinol binding subunit of the cytochrome *o* oxidase complex. CyoB is a very large heme-binding membrane protein of 74 kDa with 15 predicted TM domains, whereas CyoC is a smaller membrane protein of 20 kDa with 5 TM domains and an unknown function. Our current study deals with pre-CyoA, but *in vivo*, insertion of the subunits and their assembly into the cytochrome *o* oxidase complex is likely a coordinated process that also involves timely incorporation of the various co-factors. It will be a major challenge to elucidate the exact mechanism by which this energy-transducing complex assembles.

Experimental procedures

Strains and Plasmids

E. coli strain SF100 was used for the isolation of inner membrane vesicles (IMVs) and for overexpression of SecYEG and YidC [275]. The S135 lysate was prepared from *E. coli* MC4100. Plasmids pBSKftsQ [276] and pET27bCyoA (generous gift from Dr. M. Lübben, Department of Biophysics, Ruhr-Universitat-Bochum) were used for *in vitro* transcription of FtsQ and CyoA, respectively.

In Vitro Transcription, Translation, and Insertion Reaction

In vitro transcription was performed using the RiboMax® kit (Promega) with plasmids pBSKftsQ and pET27bCyoA as templates. *In vitro* translation-insertion reactions were performed as described [276] except that the reaction was coupled to the transcription and performed for 40 min at 37 °C.

Other Methods

IMVs containing overproduced SecYEG or YidC were isolated as described [271]. SecYEG [271], YidC [275], and SecA [36] were purified and reconstituted into *E. coli* phospholipids (Avanti Polar Lipids, Alabaster, AL) at 1 μg of SecYEG and 3 μg of YidC per 40 μgof lipids using Bio-Beads SM-2 (Bio-Rad) [275]. Proteoliposomes were analyzed by SDS-PAGE and silver staining to verify the reconstituted levels of SecYEG and YidC [275]. Functional levels of reconstituted SecYEG and YidC were verified by proOmpA translocation [275] and F_oc membrane insertion [274] assays, respectively. SecA was removed from the S135 lysate by immunodepletion and verified by immunoblotting using monoclonal SecA antibodies [276].

Acknowledgements

We thank Mathias Lübben and Martin Peter, Department of Biophysics, Ruhr-Universitat-Bochum, for donating plasmid pET27bCyoA and Martin van der Laan and Jeanine de Keyzer for discussion.

The lateral gate of SecYEG opens during protein translocation

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Summary

The SecYEG translocon of *Escherichia coli* mediates the translocation of preproteins across the cytoplasmic membrane. Here, we have examined the role of the proposed lateral gate of the translocon in translocation. A dual cysteine crosslinking approach allowed the introduction of crosslinker arms of various lengths in between adjoining amino acyl positions of trans-membrane segments 2b and 7 of the lateral gate. Oxidation and short spacer linkers that fix the gate in the closed state abolished preprotein translocation, while long spacer linkers support translocation. The crosslinking data further suggests that SecYEG lateral gate opening and activation of the SecA ATPase are coupled processes. It is concluded that lateral gate opening is a critical step during SecA-dependent protein translocation.

Introduction

Translocation of preproteins across the cytoplasmic membrane in *Escherichia coli* is mediated by the Sec translocase [For a recent review see [65]]. Preproteins targeted for secretion contain a signal sequence that is removed upon translocation. Their synthesis and translocation are uncoupled events [219], and directly after synthesis at the ribosomes, preproteins are targeted post-translationally to the Sec translocase by the molecular chaperone SecB [82]. SecB transfers the preprotein to the motor protein SecA bound at the SecYEG pore complex [36,100]. SecA utilizes cycles of ATP binding and hydrolysis to bind and release the translocating protein resulting in its step-wise translocation across the membrane [236,258,281]. In addition, the proton motive force (PMF) also facilitates translocation when the preprotein is released by SecA [63,236]. Various models for SecA mediated translocation have been proposed wherein SecA functions as power-stroke device [201] or as a directed molecular ratchet wherein SecA controls the opening and closure of the pore [170]. Another view is that SecA thrusts deep into the SecYEG channel during translocation [74,75]. In a recent study on the co-crystallization of the *Thermotoga maritima* SecA with SecYEG, it was suggested that a two-helix finger from the helical scaffold domain of SecA inserts into the cytoplasmic domain of SecY, utilizing cycles of ATP hydrolysis to push the substrate into the SecY pore [311].

The translocation pore consists of three integral membrane proteins SecY, SecE and SecG as subunits [34], and this organization is universally conserved in all three kingdoms of life [209]. The crystal structure of *Methanocaldococcus jannaschii* [270] demonstrates that the largest subunit, SecY, consists of an N- and C-terminal domain that comprise TMs 1-5 and TMs 6-10, respectively. These two domains are organized

as a clamshell-like structure that encompassses an hourglass shaped central pore. This putative pore is closed at the periplasmic face of the membrane by a short transmembrane helix, TM2a, which has been proposed to function as a plug domain. The clamshell-like structure of SecY is embraced by SecE that in its minimal form consists of a surface localized amphiphatic helix and a highly tilted transmembrane segment that localizes to the 'back' of the SecY protein. It has been proposed that the 'front' of SecY creates a lateral opening of the central pore to the membrane between TM2b and TM7 and that this gate is used to release signal sequences and transmembrane segments from the translocase [270]. Cryo-electron microscopy of the *E. coli* SecYEG complex bound to a translating ribosome [171] suggests that the ribosome bound SecYEG is organized as a dimer with a front-to-front organization [171]. It was proposed that individual pores of the dimer have distinct functions in protein translocation, i.e., vectorial protein translocation and lateral release of TMs into the membrane [169]. Freeze-fracture rotational shadowing electron microscopy has provided evidence for oligomeric forms of SecYEG, and suggest that SecA recruits SecYEG monomers to form a dimeric complex [235]. Within this dimeric SecYEG complex, only a single pore seems sufficient for the translocation of preproteins [201].

The mechanism by which the translocase coordinates protein translocation is only poorly understood. SecA has been proposed to insert the signal sequence into the SecYEG pore where it may latch in between TM2b and TM7 of the SecY lateral gate. This would result in a widening of the central pore constriction and a subsequent displacement of the periplasmic plug domain. Next, adjoining polypeptide segments of the preprotein may enter the opened aqueous pore, but it is not clear if under those conditions the lateral gate remains open or is closed. Despite this vast amount of

experimental data available on the function of the SecYEG complex, the exact role of the putative lateral gate remains unknown. Thus far, the only study on its dynamics and role during translocation concerns a molecular dynamics simulation [94] that does not take SecA or ribosome binding into account. Here we have investigated the function and the dynamics of the proposed lateral gate located between TM2b and TM7 in protein translocation. The data demonstrates that the lateral gate needs to open to allow for SecA-mediated preprotein translocation.

Results

Introduction of cysteines in the putative lateral gate of SecYEG

To date, no direct evidence exist for the functioning of the proposed lateral gate in between TM2b and TM7 of SecY [270]. This gate has been suggested to play a role in channel opening, release of the signal sequence of translocating proteins, as well as in the insertion of transmembrane helices of nascent integral membrane proteins into the lipid bilayer [270]. To investigate the functioning of the lateral gate we identified amino acid positions in TM2b and TM7 in *E. coli* SecY (**Fig. 1**A) that based on the *M. jannaschii* SecYEβ crystal structure [270] (**Fig. 1**B) would be within disulfide bonding distance of each other. As controls, we also selected amino acids that are predicted to be too far apart to form a disulfide bond. The selected amino acids were replaced by cysteine residues via site directed mutagenesis using a cysteine-less (Cys-less) SecY as template. Subsequently, the various single cysteine mutants in TM2b were combined with single cysteine mutants in TM7 to form double cysteine mutant pairs (**Table 1**). From the possible combinations, we specifically selected the pair

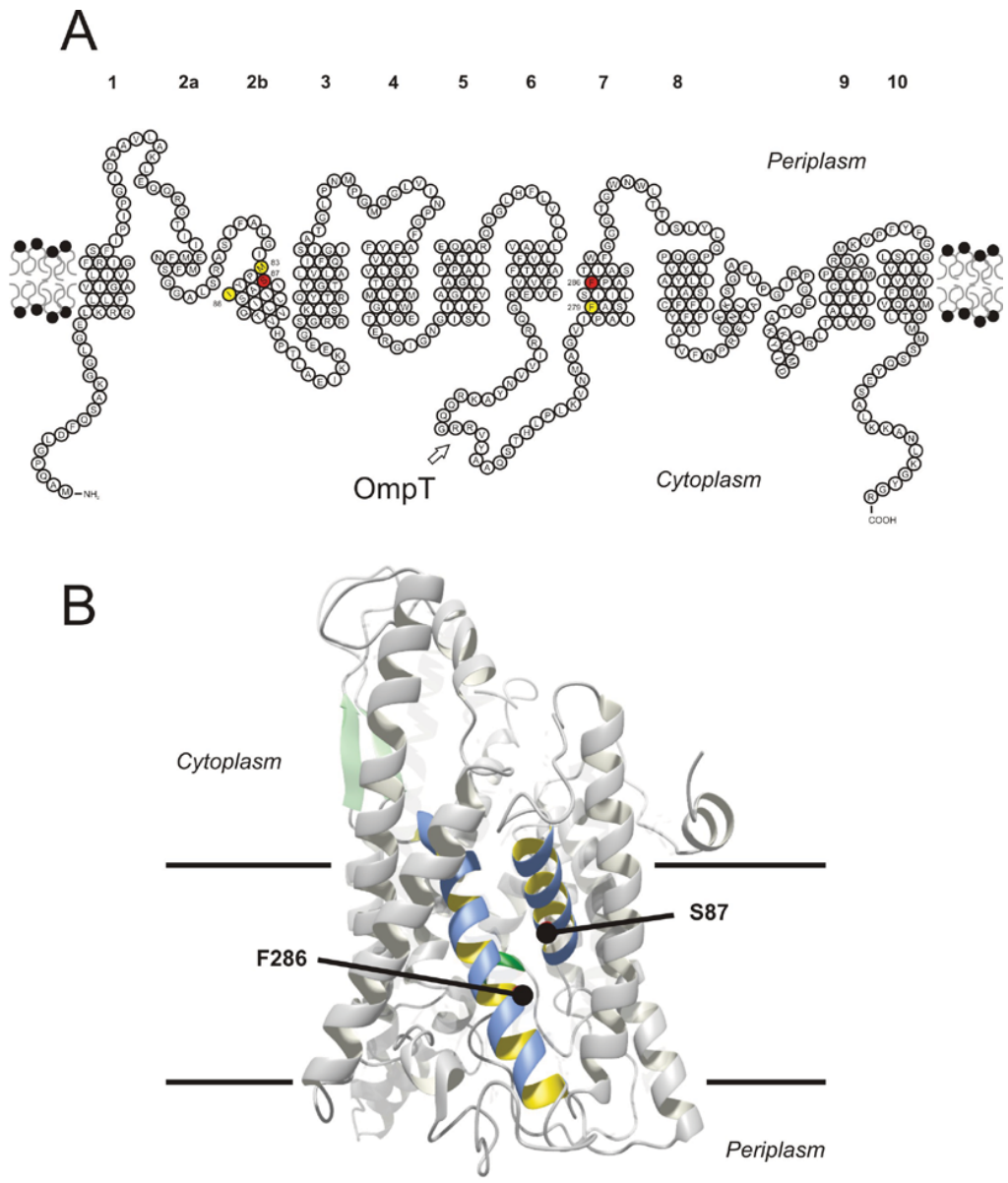

Fig. 1 Introduced cysteine mutations in TM2b and TM7 of the lateral gate of SecY. (A) Topology model of *E. coli* SecY based on a sequence alignment with the *M. jannaschii* SecY crystal structure [270]. Highlighted are the TM segments 2b and 7 and the amino acids residues selected for cysteine mutagenesis (red, S87 and F286; yellow, M83, I86, and F279). Also indicated is the OmpT cleavage site in cytoplasmic region C4. (B) Side view of the *M. jannaschii* SecY crystal structure showing the putative gate region [1RHZ.pdb; [270]]. Indicated are amino acids S87 and F286 in TM2b and TM7, respectively. The figure was created using MOLMOL [144] and POV-Ray (www.povray.org).

F286C/S87C, as the homologous amino acids (N268 and T80, respectively) in the *M. jannaschii* SecYEβ form hydrogen bonds that need to be broken to open the lateral gate [270] (**Table 1**). The various SecY mutants were cloned into a *secYEG* expression vector and expressed in *E. coli* strain SF100. SDS-PAGE analysis and

immunoblotting using an antibody against the N-terminal histidine tag in SecY showed that all mutants were (over)expressed at similar levels (See **Fig. 2** and Supplemental **Fig. S2**).

In vitro translocation experiments using fluorescein (FL) labeled proOmpA as substrate and IMVs from cells overproducing the different single and double cysteine mutants showed that under reducing conditions all mutants translocate proOmpA to similar levels as the Cys-less SecYEG (Supplemental **Fig. S1**).

Chemical crosslinking of the putative lateral gate formed by TM2b and TM7

To visualize the crosslinking between cysteines introduced in TM2b and TM7, an assay was developed based on the specific cleavage of SecY by the outer membrane protein OmpT. OmpT cleaves SecY in the fourth cytoplasmic domain (C4) between two arginine residues at positions 255 and 256 (**Fig. 1**A) [5]. Cleavage results in a N-terminal fragment of SecY with a apparent molecular mass of 22 kDa that is readily detected by CBB-stained SDS-PAGE (**Fig. 2**A and Supplemental **Fig. S**2). When TM2b is crosslinked to TM7, OmpT will cleave SecY but the two halves will not be separated on non-reducing SDS-PAGE and SecY will migrate as a full length protein.

To oxidize the cysteine pairs in SecY, the hydrophilic oxidizer sodium tetrathionate (NaTT) was used to form a disulfide bond. The use of the hydrophobic oxidizer Cu-Phenantroline was avoided as this agent can cause protein aggregation, while there is the risk of a formation of thiol-Cu-thiol bridge rather than a disulfide bond. Dithiothreitol (DTT) was used as a reducing agent to reverse disulfide bond formation. After NaTT-treatment, IMVs were incubated with the protease OmpT and analyzed by SDS-PAGE and CBB staining (**Fig. 2**A). Under both

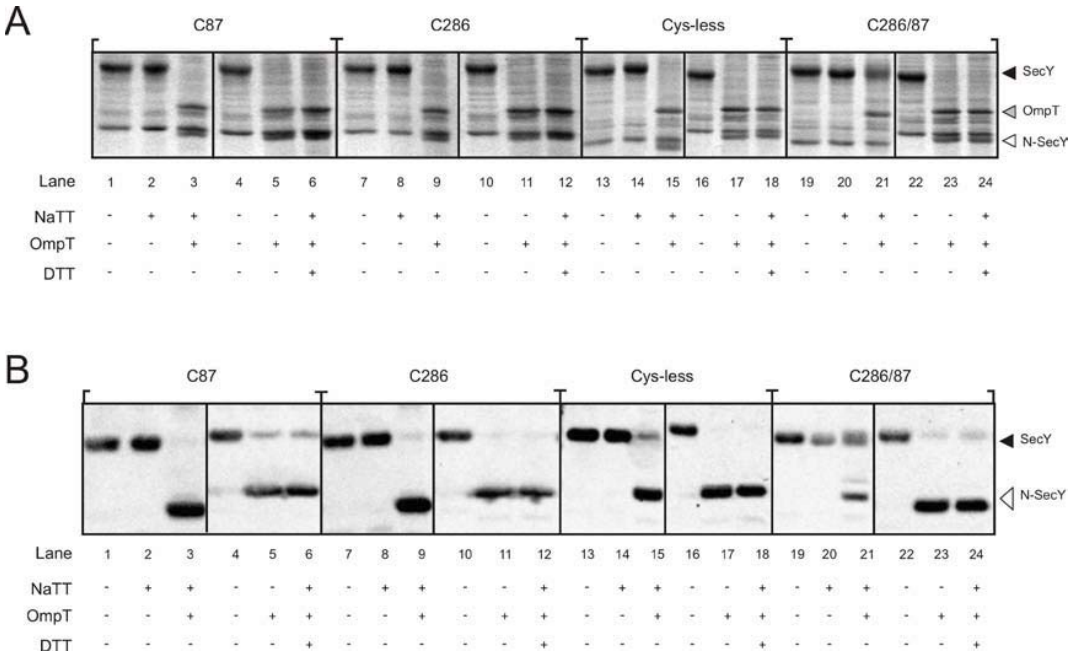

Fig. 2 Disulfide crosslinking of cysteines in the lateral gate formed by TM2b and TM7. (A) IMVs containing different SecYEG derivatives were treated with the oxidizer sodium tetrathionate (NaTT, 1 mM). To access the crosslinking efficiency of the cysteine residues in SecY, the IMVs were incubated with the protease OmpT and analyzed by CBB-stained SDS-PAGE. (B) Immunostaining of the SDS-PAGE using an antibody against the N-terminal six histidine tag in SecY. Note, to prevent the reduction of the disulfide bond during electrophoresis, samples which contained DTT were analyzed on a different gel than samples without DTT. For this reason there is a slight difference in gel mobility of full length SecY and N-SecY. On the same gel, the N-SecY fragments of all SecY mutants migrate at the same position.

oxidizing and reducing conditions, OmpT treatment of the IMVs containing Cys-less SecY resulted in the complete cleavage of SecY as seen as a disappearance of full length SecY with the concomitant formation of the 22 kDa N-terminal fragment (**Fig. 2**A, *lanes 15* and *17*). Moreover, addition of the reducing agent DTT after OmpT digestion (**Fig. 2**A, *lane 18*) did not affect the cleavage pattern (**Fig. 2**A, compare *lane 17* vs. *18*). It should be noted that samples containing DTT were loaded on a separate SDS-PAGE gel in order to prevent the DTT from diffusing and affecting neighboring crosslinked samples. For this reason there is a slight difference in migration of full length SecY and derived N-terminal fragment of the same samples with and without DTT (See **Fig. 2**). The same results as shown for Cys-less SecY were obtained with all single cysteine mutants (**Fig. 2**A, *lanes 1-12* and Supplemental **Fig. S**2, *lanes 1-*

18). In contrast, after oxidation of IMVs containing the double cysteine mutant SecY C286/87, OmpT treatment resulted in the formation of a fuzzy band that corresponds to full length SecY without appearance of the 22 kDa N-terminal fragment. This was not due to an incomplete digestion or NaTT-induced protein aggregation, as addition of DTT to the sample after the OmpT treatment resulted in the conversion of full length SecY into the 22 kDa N-terminal fragment (**Fig. 2**A, lane 24). This indicates that NaTT treatment results in the formation of a disulfide bond between the selected cysteine pair 286 and 87. The fuzzy character of the protein band corresponding to OmpT cleaved SecY is noted, however, full length SecY indicates an altered conformational state of SecY in SDS-PAGE, a phenomenon often seen in crosslinking of membrane proteins. In comparison with the other double cysteine mutants, disulfide bond formation was very efficient with the mutant C286/87 and weaker for the C279/86 (compare **Fig. 2**A, *lanes 19-24* vs. Supplemental **Fig. S2**, *lanes 19-30*) and C283/87 (data not shown) pairs. Crosslinking was absent with the negative control, mutant C279/83 (Supplemental **Fig. S2**, *lanes 19-24*) confirming the SecYEG structure that indicated that these residues are not within disulfide bonding distance. For this reason, the SecY C286/87 mutant was used in the remainder of this study.

To verify the oxidation and OmpT digestion, formation of the N-terminal 22 kDa SecY fragment was validated by Western blotting using an antibody directed against the six-histidine tag (**Fig. 2**B). OmpT treatment of NaTT oxidized IMVs containing Cys-less SecYEG and single cysteine mutants resulted in the complete conversion of SecY into the 22 kDa N-terminal fragment (**Fig. 2**B *lanes 1-18*). On the other hand, in oxidized IMVs containing the C286/87 mutant, substantial levels of intact SecY remained after OmpT treatment (**Fig. 2**B, *lane 21*). The full length SecY was

converted into the 22 kDa N-terminal SecY fragment after DTT treatment (**Fig. 2**B, *lane 24*). The small amount of N-SecY fragment in **Fig. 2**B *lane 21* indicates incomplete disulfide bond formation. However, since the blotting transfer of full length SecY, the N-terminal fragment and the crosslinked products likely differ, data quantification was based on the full length SecY visualized by SDS-PAGE. NaTT mediated oxidation of the C286/87 SecY mutant pair results in a crosslinking efficiency of at least 80 % (Refer to **Fig. 4**A*, lane 4* for crosslinking efficiency).

Oxidative crosslinking of the lateral gate abolishes proOmpA translocation and SecA Translocation ATPase activity

To determine if crosslinking of the cysteines in the SecY C286/87 mutant has an effect on protein translocation, we analyzed the *in vitro* translocation of FL-labeled proOmpA into IMVs that had been oxidized with NaTT or reduced with DTT. With IMVs containing Cys-less SecY, NaTT treatment had little effect on proOmpA translocation (**Fig. 3**A, compare *lanes 3* and *4*), nor did it affect proOmpA translocation into IMVs containing the SecY single cysteine C286 (**Fig. 3**A, *lane 10*) and C87 (**Fig. 3**A, *lane 8*), although the latter showed a reduced activity which can be attributed to the mutation. In contrast, NaTT treatment of IMVs containing the double cysteine mutant C287/87 was nearly completely abolished to the very low levels found for the endogenous wild-type levels of SecYEG (**Fig. 3**A, compare *lane 6* vs. *2*). The activity of the C279/86 mutant was reduced by a lesser extent by NaTT oxidation in line with the observed weaker disulfide bonding efficiency (Supplemental **Fig. S3** *lanes 3-4*). As expected, proOmpA translocation into NaTT treated IMVs containing the negative control SecY C279/83 was not affected

(Supplemental **Fig. S3**, *lanes 5-6*). These conditions also do not lead to the formation of a disulfide bond between the two positions.

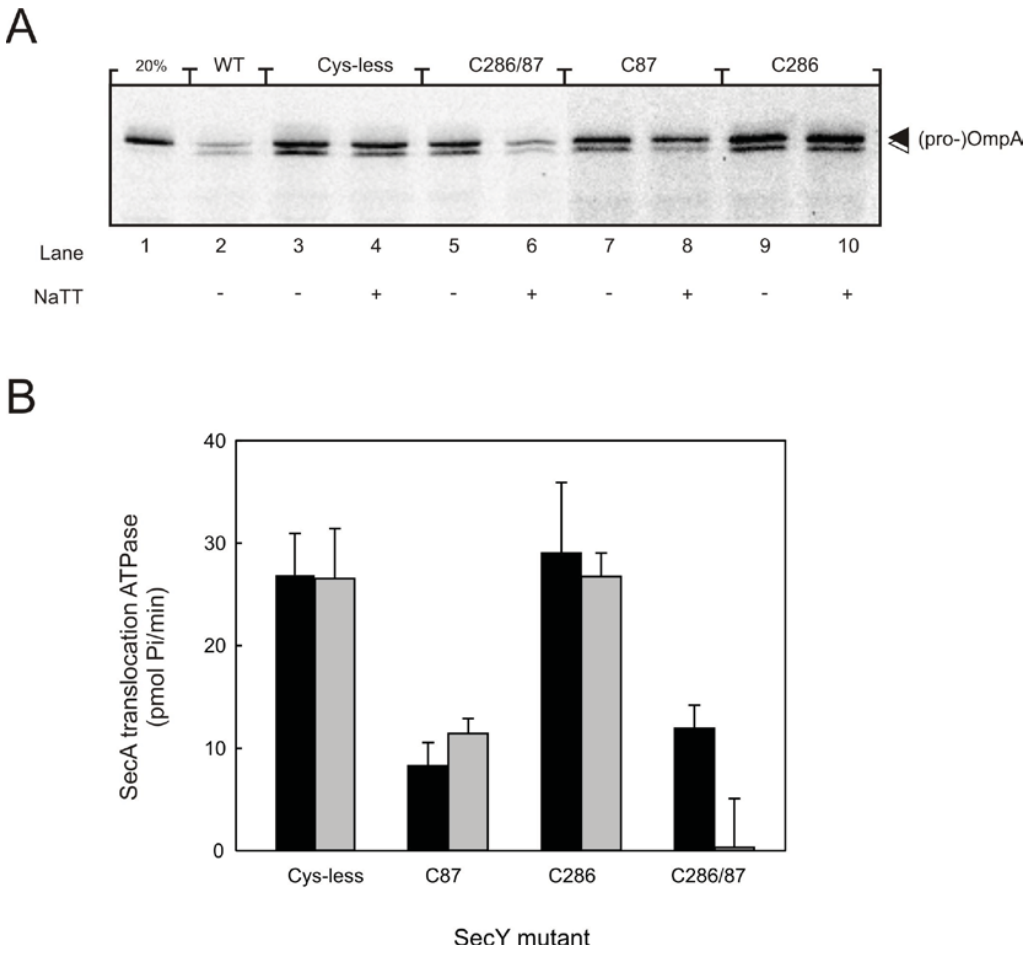

Fig. 3 An intra-molecular disulfide bond in SecY C286/87 inhibits proOmpA translocation. (A) Translocation of FL-proOmpA into wild-type IMVs (lane 2) or IMVs containing overexpressed levels of Cys-less (lanes 3-4), C286/87 (lanes 5-6), C286 (lanes 7-8) or C87 (lanes 9-10) SecYEG. Prior to translocation, IMVs were oxidized or reduced with NaTT (lanes 4, 6, 8, and 10) and DTT (lanes 2, 3, 5, 7 and 9), respectively. Lane 1 shows a 20 % FL-proOmpA standard. (B) SecA Translocation ATPase activity of IMVs containing Cys-less, C286/87, C286 or C87 SecYEG and that had been pre-incubated with (black bars) or without (white bars) NaTT.

Since proOmpA-translocation is a SecA-dependent process, the effect of the lateral gate crosslinking on the proOmpA-stimulated ATPase activity was determined. IMVs containing the Cys-less, single cysteine C87 and C286, and the double cysteine C286/87 SecYEG mutant were treated with NaTT, and analyzed for the proOmpA stimulated SecA translocation ATPase activity. NaTT treatment of IMVs containing Cys-less SecYEG did not significantly affect the SecA translocation ATPase activity

(**Fig. 3**B). Similar results were obtained with IMVs containing the single cysteine mutants SecY C87 and C286, although the activity of the SecY C87 mutant was reduced as compared to the Cys-less control. The latter explains the reduced proOmpA stimulated ATPase activity of the untreated double cysteine mutant SecY C286/87 (**Fig. 3**B), and possibly signifies a functional role of S87 in translocation. Importantly, upon NaTT treatment of IMVs containing the double cysteine mutant SecY C286/87, the proOmpA stimulated ATPase activity of SecA was completely abolished. These data demonstrate that immobilization of the lateral gate of the translocon by disulfide-bonded crosslinking inhibits SecA mediated preprotein translocation and the SecA translocation ATPase activity.

Immobilization of the lateral gate of SecY by chemical crosslinkers with varying length

NaTT forms a disulfide bond between the two cysteine residues with an approximate distance of 2 Å. Oxidation is reversible by the use of the reductant DTT. To analyze the effect of the crosslinking distance of the two cysteines on protein translocation, chemical crosslinkers were employed that have spacer arms with increasing lengths (Supplemental **Fig. S5**). Dibromobimane (bBBr) forms a rigid covalent bond between thiol groups and has a crosslinking distance of 5 Å [146,147]. bBBr has the particular characteristic that it becomes fluorescent when both of its alkylating groups have reacted with thiols [134]. Bis-maleimidoethane (BMOE) is a rigid crosslinker that contains a thioether-linked spacer of ~8 Å while bis-maleimidohexane (BMH) is a longer yet more flexible crosslinker with a maximal ~13 Å spacer arm [40]. With the SecY C286/87 mutant, bBBr showed similar

crosslinking efficiency as NaTT at 84 and 81%, respectively (**Fig. 4**A, compare *lane 5* and *4*). The crosslinking efficiencies of the longer crosslinking molecules BMOE and BMH were somewhat lower, i.e., 56 and 59%, respectively (*lane 6* and *lane 7*) but clearly discernable upon OmpT-treatment. None of these crosslinkers interfered with the efficiency of OmpT digestion as validated with the Cys-less SecYEG complex digested by OmpT in the presence of these compounds (**Fig. 4**A, *lanes 9-13*). In addition, the crosslink between cysteines 286 and 87 after bBBr treatment could be directly demonstrated by UV exposure of a SDS-PAGE gel showing a bright fluorescent band at the position of full-length SecY with the C286/87 mutant (**Fig. 4**B, *lane 4*) and that is absent in the bBBr treated single-cysteine and Cys-less mutants (*lanes 1-3*). The direct fluorescent visualisation provides further evidence for the formation of a crosslink between C286 and C87 in addition to the OmpT assay.

IMVs containing the Cys-less and C286/87 mutations were treated with the crosslinker NaTT, bBBr, BMOE and BMH as well as with the reductant Tris[2-carboxyethyl] phosphine (TCEP), respectively. TCEP instead of DTT was used here as it is capable of reducing thiol in the presence of reactive maleimides. Next, *in vitro* translocation assays were performed using Texas Red (TR) labeled proOmpA as substrate. TR-proOmpA has an emission spectrum that does not overlap with the emission spectrum of bBBr, thus allowing for the simultaneous detection of proOmpA translocation and crosslinking of the lateral gate in SecY C286/87. At the limiting amounts of IMVs used, the activity of SecYEG is rate-determining for translocation (Supplemental **Fig. S4**) and thus the activity can be quantified and compared. None of the chemical crosslinkers affect the translocation of proOmpA into the Cys-less SecYEG IMVs (**Fig. 4**C, *lanes 8-12*). As shown before, NaTT treatment of SecY

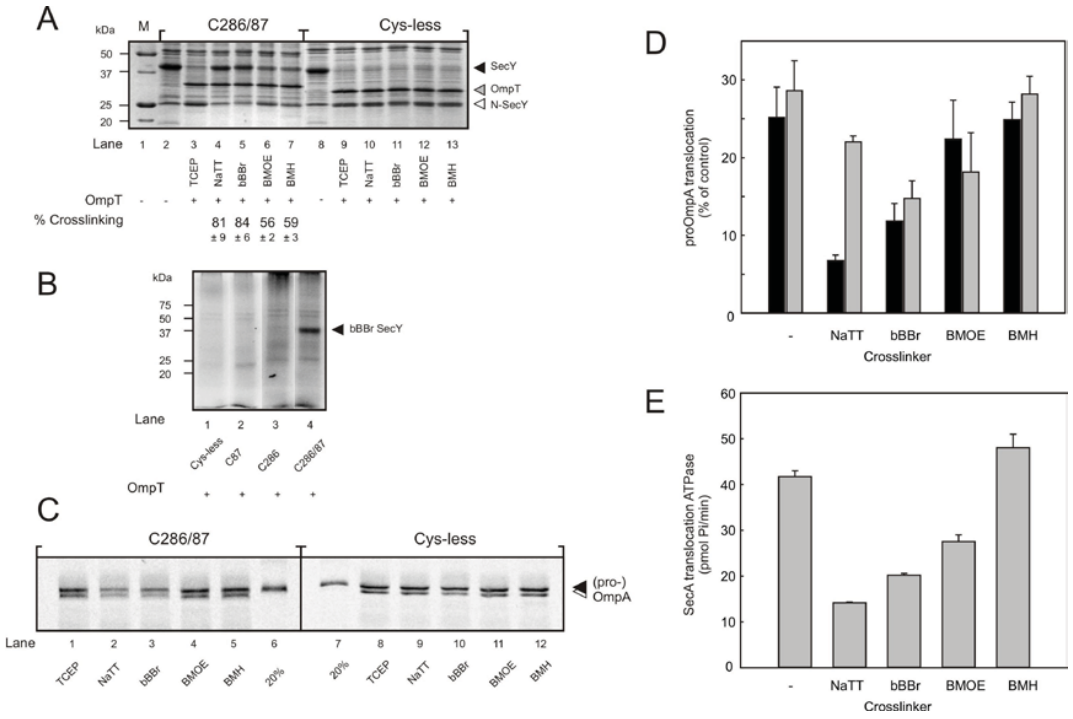

Fig. 4 Crosslinking of SecY C286/87 with thiol reactive reagents of different spacer lengths. (A) IMVs containing Cys-less or C286/87 SecYEG were incubated with the reducing agent TCEP, the oxidizer NaTT (2 Å), or the cross-linkers dibromo bimane (bBBr; 5 Å), bis(maleimido)ethane (BMOE, ~8 Å) and bis(maleimido)hexane (BMH, ~13 Å). After 30 minutes at 37°C, IMVs were treated with OmpT and the crosslinking of the cysteine residues in SecY was analyzed by CBB-stained SDS-PAGE. Crosslinking efficiencies were calculated from a minimum of three independent gels using full length SecY band in lane 2 as 100% control, and lane 3 as background. (B) Direct fluorescent monitoring of the cross-linking of C87 and C287 of SecY by bBBr. IMVs containing Cys-less, C87, C286 or C286/87 SecYEG were incubated with bBBr. After 30 minutes at 37°C, IMVs were treated with OmpT and the cysteine crosslinking was analyzed by SDS-PAGE and in gel UV fluorescence using a cut-off filter of 520 nm. (C) Translocation of TR-proOmpA into IMVs containing Cys-less (lanes 1-5) or C286/87 (lanes 8-12) SecYEG that had been incubated with different crosslinkers. Lanes 6 and 7 show a 20% TR-proOmpA standard. (D) Quantification of the TR-proOmpA translocation into IMVs containing C286/87 SecYEG treated with different crosslinkers. After crosslinking, the IMVs were incubated with (grey bars) or without (black bars) the reductant DTT, and used for translocation assays as described in the Experimental Procedure section. (E) SecA Translocation ATPase activity in the presence of IMVs containing C286/87 SecYEG treated with different crosslinkers. Values were corrected for the ATP hydrolysis in the absence of preprotein.

C286/87 IMVs reduced translocation to the levels observed with wild-type IMVs containing only endogenous levels of SecYEG (**Fig. 3**A, *lane 6*; **Fig. 4**C *lane 2*). A strong reduction in activity was also observed when SecY C286/87 IMVs were treated with bBBr (**Fig. 4**C, *lane 3*), while the longer crosslinkers BMOE and BMH had little effect on proOmpA translocation (**Fig. 4**C *lane 4* and *5*). Quantification of the data shows that the translocation efficiency increases with an increasing length of the

spacer arm reaching the same level of activity as untreated IMVs with the longest crosslinker BMH (**Fig. 4**D, black bars).

We also addressed the reversibility of the oxidation and crosslinking. Addition of DTT after the crosslinking reaction restored translocation to normal levels only for the NaTT-oxidized C286/87 IMVs (**Fig. 4**D, grey bars). As expected, DTT had no significant effect on the translocation of proOmpA into IMVs treated with the irreversible crosslinkers bBBr, BMOE and BMH. It should be noted that none of these compounds affected the activity of the single C87 mutant (Supplemental **Fig. S6**). Thus the inhibition of proOmpA translocation is due to a constrained lateral gate and is not caused by alkylation of a single cysteine position.

Next we performed SecA ATPase assays using SecY C286/87 IMVs treated with the various crosslinkers. Whereas these compounds did not affect the basal SecA ATPase activity of the IMVs (data not shown), the proOmpA stimulated ATPase activity increased with the length of the spacer arm of the crosslinker (**Fig. 4**E) matches their effect on proOmpA translocation. Although the reduced efficiency of the BMH crosslinking will contribute to the observed translocation and SecA translocation ATPase activities, it does not explain the entirely undisturbed activities with the BMH crosslinked C286/87 SecY (**Fig. 4**D and **Fig. 4**E). Threfore, these data lend strong support for the notion that SecYEG lateral gate opening and the activation of the SecA ATPase are linked processes.

Crosslinking of the lateral gate of a translocon containing a protein translocation intermediate

A pre-existing disulfide bond between positions 286 and 87 of the lateral gate of SecY prevents preprotein translocation. To access the proximity of these positions during translocation, a translocation intermediate was generated using a proOmpA-dihydrofolate reductase (DHFR) fusion protein [9,28,124]. Upon addition of the ligands NADPH and methotrexate, the DHFR moiety folds and blocks further translocation. This yields a translocation intermediate that is stably arrested in the translocase. To ensure that proOmpA-DHFR efficiently blocks the C286/87 SecYEG pore, translocation was performed with proOmpA-DHFR in excess of the translocation sites whereupon IMVs were re-isolated and incubated with TR-proOmpA for a second round of translocation. Unlabeled proOmpA translocated during the first incubation period did not interfere with subsequent translocation of TR-proOmpA (**Fig. 5**A, *lane 2*). However, the folded DHFR domain in the proOmpA-DHFR fusion protein nearly completely blocked the translocation sites as translocation of TR-proOmpA in the second round was greatly reduced (**Fig. 5**A, *lane 3*). Next, the SecYEG C286/87 IMVs loaded with the proOmpA-DHFR translocation intermediate were incubated with different chemical crosslinkers and the formation of a bond between C286 and C87 was analyzed using the OmpT protease assay and gel staining with SYPRO® Ruby. Oxidation with NaTT or addition of the short crosslinker bBBr to the IMVs containing the translocation intermediate yielded similar levels of crosslinked SecY as observed with the unoccupied pore (**Fig. 5**B, *lanes 3* and *4*, compare with **Fig. 4**A, *lanes 4* and *5*). This demonstrates that the cysteines are within disulfide bonding distance when the pore is occupied with a

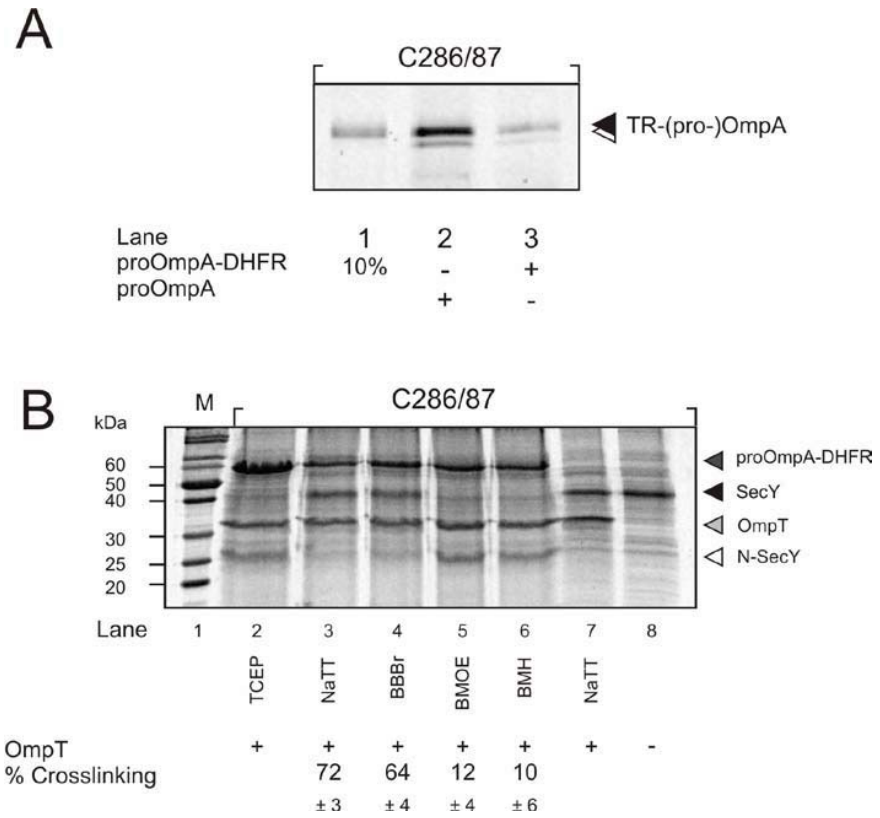

Fig. 5 C286 and C87 are in close proximity when a translocation intermediate is arrested in the SecYEG pore. (A) A proOmpA-DHFR translocation intermediate blocks the translocation sites of IMVs containing C286/87 SecYEG. Unlabeled proOmpA-DHFR (800 μg/ml; supra stoichiometric to the number of translocation sites) and 10 mM NADPH + 10 μM methotexrate (lane 2) or proOmpA (lane 3) was incubated with C286/87 SecYEG IMVs (250 μg/ml) and SecA (20 μg/ml) for 10 minutes at 37°C in the presence of 2 mM ATP. Membranes were re-isolated by ultracentrifugation, resuspended in translocation buffer with SecA (20 μg/ml), 2 mM ATP (and 10 mM NADPH and 10 μM methotexrate for proOmpA-DHFR), and assayed for a second round of translocation using TR-proOmpA. Lane 1 shows a 10 % TR-proOmpA standard. (B) C286/87 SecYEG IMVs charged with the proOmpA-DHFR translocation intermediate were incubated with the indicated crosslinkers. After 30 minutes at 37°C, the protease OmpT was added to determine the amount of crosslinked SecY. Gels were stained with SYPRO® Ruby. The positions of proOmpA-DHFR, SecY, OmpT and the N-terminal fragment of SecY are indicated by arrows. Lanes 7 and 8 are controls showing the NaTT-oxidized C286/87 SecYEG IMVs treated with OmpT, and DTT-reduced C286/87 SecYEG IMVs, respectively. The crosslinking efficiency was calculated from three independent gels using the full length SecY band in lane 8 as 100% control, and lane 2 as background.

translocation intermediate. On the other hand, crosslinking with the longer spacer length BMOE and BMH was very inefficient with a dramatic reduction of the amount of full length SecY upon OmpT treatment concomitant with the formation of a prominent 22 kDa SecY fragment (**Fig. 5**B, *lanes 5* and *6*). This is in marked contrast, to the efficiency of crosslinking in the absence of a translocation intermediate (See **Fig. 4**A, *lanes 6* and *7*). These data demonstrate that C87 in TM2 and C286 in TM7

are in close proximity when the pore is occupied by a translocation intermediate. Likely because of geometrical constraints, these positions can no longer be crosslinked by the chemical reagents with longer spacer arms.

Discussion

In this study, we have addressed the role of the lateral gate in the translocation of preproteins across the cytoplasmic membrane. On the basis of homology and sequence alignment with *M. jannaschii* SecY [270], we have engineered pairs of cysteine residues into the putative TM2b/TM7 lateral gate region of the *E. coli* SecY. This allowed site-specific disulfide-bonded cross-linking of positions that were predicted to be in close vicinity, whereas more remotely introduced cysteines did not yield crosslinks. Recently, two additional SecYE translocon structures have been described, notably from bacteria known to contain SecA as opposed to the archaeon *M. jannaschii* [259,311]. The high resolution structure of SecYE from *Thermus thermophilus* is in a antibody-stabilized pre-open state [259] while the structure of SecYEG from *Thermotoga maritima* is with SecA bound in an intermediate state of ATP-hydrolysis [78]. Strikingly, compared to the *M. jannaschii* SecYEβ structure, the *T. maritima* SecA-bound SecYEG structure shows a partial opening of the lateral gate region around TM2b and TM7. Our biochemical crosslinking data for *E. coli* SecYEG on the lateral gate region are in line with this structural observation, and importantly, we demonstrate that the opening of this lateral gate is required for protein translocation and activation of the SecA ATPase.

The introduction of double cysteine mutants in TM2b and TM7 allowed an efficient crosslinking of the lateral gate. When fully oxidized, translocation is

completely abolished. However, when a crosslinker is introduced with sufficient spacer length (larger than 5 Å), translocation occurred unrestricted. These observations support two main conclusions: i) translocation of proOmpA occurs via a single translocon in line with a previous report [201]; and ii) the lateral gate region needs to open up to allow translocation to take place. It should be noted that the formation of a functional consolidated pore between two opposing SecYEG translocons seems unlikely because the presence of for instance the BMH crosslink in the lateral gate of one of the pores will prevent diffusion of the polypeptide substrate to the other pore.

Strikingly, there is a strong cooperativity between the ability of SecA to hydrolyse ATP and the opening of the lateral gate. Oxidative crosslinking of the lateral gate resulted in a complete loss of the SecA translocation ATPase activity, whereas chemical modification of the lateral gate with bimolecular crosslinkers with longer spacer arms (8-13 Å) supported SecA translocation ATPase activity up to the level observed with an unconstrained lateral gate. A recent study on the role of the *E. coli* SecA ATPase two-helix finger suggested that the helical scaffold domain (HSD), that harbors the two-helix finger, moves up and down inside the translocon with ATP hydrolysis cycles resulting in the pushing of the preprotein substrate into the translocon [78]. Two cysteine positions on proOmpA could be crosslinked to both position 282 of SecY together with a cysteine on the tip of the two-helix finger [78]. This is very close to the crosslinked positions 286 and 87 of the lateral gate as shown in this study. This further highlights the importance of the region. Tsukazaki *et al* [259] suggested that SecYE predominantly assumes the closed conformation while binding of SecA induces a conformational change of the translocon from the closed to the pre-open state where a swinging of TM8 possibly induces an opening of the lateral

gate region. Taken together, our data shows that a tight physical interaction between TM2b and TM7 is incompatible with preprotein translocation lending strong support for the hypothesis that the clamshell structure of the SecYEG translocon needs to open for the SecA-dependent initiation of protein translocation.

A recent molecular dynamic simulation study modeled conformational changes in SecYEβ upon translocation initiation, where a force of 2-3 nN is required to move TM2b and TM7 apart [94]. Two independent events are proposed to require this amount of force. The first event is the opening of the gate to around 2 to 5 Å which involves a latch-like movement of SecE and which requires a large force. The second event is the displacement of the plug domain (TM2a) which allows the lateral gate to open to 6-9 Å. Crosslinking studies suggested that the signal sequence from a translocating protein binds the region between TM2b and TM7 [207] and based on this observation it was proposed that the signal sequence inserts in between TM2b and TM7 resulting in a displacement of the plug and the concomitant translocation of the mature preprotein domain. Our biochemical data are consistent with the MD simulation as a SecY channel in which the gate is fixed by a crosslinker with a 8 Å or longer spacer arm allows normal translocation of proOmpA, whereas a crosslinker of 5 Å or shorter abolishes protein translocation. Interestingly we also observed that TM2b and TM7 can be efficiently crosslinked utilizing the oxidizer NaTT and the short crosslinker bBBr even when the pore contains a preprotein translocation intermediate. As the major fraction of the preprotein translocation intermediate is processed by leader peptidase [124] (data not shown), the signal sequence is no longer present in the complex. In contrast, the longer crosslinkers BMOE and BMH were no longer able to crosslink the cysteine positions in TM2b and TM7 when the pore was occupied by a translocation intermediate, despite their hydrophobicity that may

facilitate access of the cysteine positions via the lipid phase. This suggests that the lateral gate region is in a near to closed conformation with the translocation intermediate present. Most likely when SecA encounters the DHFR domain at the C-terminus of the proOmpA-DHFR fusion, SecA remains in an ADP-bound state and is unable to unfold the DHFR domain [193]. The latter may correspond to a state of the SecA ATPase in which the two-helix finger is not inserted into SecY [78]. Possibly, under these conditions the SecA-SecY interaction corresponds to a more energetically favorable pre-open state [259]. In the *T. maritima* SecA-SecYEG complex structure [311], the opening in the lateral gate between TM7 and TM2b is about 5 Å. Likely, longer crosslinkers such as BMOE and BMH will not be able to efficiently crosslink these selected positions when the flexibility of this region is constrained by the SecA transition state and/or the protein translocation intermediate. When the folding ligands of DHFR (NADPH and methotrexate) are removed further translocation of the DHFR domain occurs in an ATP and SecA-independent manner [9]. Therefore, with a stalled and processed translocation intermediate, the SecY translocon most likely returns to a pre-open state with bound SecA, which corresponds to a narrowed lateral gate region.

In conclusion, for the first time we have provided experimental evidence for a catalytic requirement for the opening of the lateral gate at the interface of TM2b and TM7 of SecY during SecA-dependent protein translocation. Importantly, SecA activation and lateral gate opening are coordinated events consistent with an allosteric mechanism of channel opening. Eventually, signal sequences may leave the translocon via the TM2b and TM7 gate. Taken together, the SecYEG translocon seems to be a highly flexible structure that undergoes specific conformational changes during protein translocation. Future studies should address the function of the lateral gate in the insertion of membrane proteins.

Experimental procedures

Chemicals and biochemicals

Purification of SecA, SecB, proOmpA and proOmpA-DHFR as well as the isolation of cytoplasmic membrane vesicles (IMVs) containing overproduced levels of SecYEG were performed as described [271]. ProOmpA (S245C) was labeled with fluorescein (FL) or Texas Red (TR) maleimide (Invitrogen^{TM}) as described [51]. The crosslinking reagents bis-maleimido ethane (BMOE) and bis-maleimido hexane (BMH) as well as the reducing agent Tris(2-Carboxyethyl) phosphine (TCEP) were obtained from Pierce (Rockford, IL, USA). Sodium tetrathionate (NaTT) was from Sigma-Aldrich and dibromobimane (bBBr) was from Invitrogen. The reducing agent 1,4-dithiothreitol (DTT) was obtained from Roche Applied Science. Enzymes for DNA manipulation were obtained from Promega, Roche and Fermentas and all other chemicals were from Sigma-Aldrich.

Bacterial strains and plasmids

All strains and plasmids used are listed in **Table 1**. All DNA manipulations were performed using *E. coli* DH5α to maintain plasmids and constructs. Cysteine mutations were introduced into a Cys-less SecY using the Stratagene QuickChange® site-directed mutagenesis kit using plasmid pEK1 as template. Mutations were confirmed by sequencing. Plasmids expressing the mutated SecYEG complex were created by exchanging the *Nco*I-*Cla*I *secY* fragment in pEK20 by the cysteine containing *Nco*I-*Cla*I *secY* fragment of the pEK1 derivative. Plasmid pET651 (A.

Kaufmann, unpublished data) harboring the F279C mutation, was used as template to generate the 279/83 and 279/86 double cysteine mutants. *E. coli* strain SF100 or NN100 (SF100, *unc*$^-$) was used for the overproduction of the various SecYEG complexes.

Table 1 Overview of strains and plasmids used in this study

Strain / Plasmid	Relevant characteristic	Source
E. coli DH5α	*supE44, ΔlacU169 (Δ80lacZ_M15) hsdR17, recA1, endA1, gyrA96 thi-1, relA1*	[96]
E. coli SF100	*F*$^-$*, ΔlacX74, galE, galK, thi, rpsL, strA, ΔphoA(pvuII), ΔompT*	[16]
E. coli NN100	SF100, *unc*$^-$	[197]
E. coli BL21 (DE3) Rosetta	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE2 (Cam^R)	Novagen
pND9	OmpT	[149]
pET80	proOmpA(C290S)-DHFR	[28]
pET36	proOmpA(S245C)	F.Bonardi, unpublished data
pEK1	Cysteine-less SecY	[279]
pEK20	Cysteine-less SecYEG	[279]
pET651	SecY(F279C)EG	A.Kaufmann, unpublished data
pFE-SecY1	SecY(M83C)EG	This study
pFE-SecY4	SecY(I86C)EG	This study
pFE-SecY5	SecY(S87C)EG	This study
pFE-SecY7	SecY(I283C)	This study
pFE-SecY10	SecY(F286C)EG	This study
pFE-SecY13	SecY(I283C/S87C)EG	This study
pFE-SecY16	SecY(F286C/S87C)EG	This study
pFE-SecY61	SecY(F279C/M83C)EG	This study
pFE-SecY62	SecY(F279C/I86C)EG	This study

OmpT was expressed from plasmid pND9 in strain SF100 and expressed under its own temperature sensitive promoter [149]. After overnight incubation at 37ºC, outer membranes containing high levels of OmpT were isolated by differential centrifugation. Briefly, cells were harvested at 7 500 rpm for 15 min in a JLA 10.500 (Beckman) rotor, resuspended in a buffer containing 50 mM Tris-HCl pH 8 and 1 mM EDTA whereupon the suspension was passed twice through a cell disruptor at 8,000 psi. Cell debris was removed by centrifuging at 4,000 rpm for 15 min in a SS-34 (Beckman) rotor. The supernatant was transferred to a Ti45 ultra centrifuge rotor

(Beckman) and centrifuged for 1 hour at 40,000 rpm and 4°C. The outer membrane pellet was resuspended in a buffer containing 50 mM Tris-HCl pH 8 and 250 mM sucrose and stored in small aliquots at -80°C.

Chemical crosslinking and OmpT assay

IMVs containing overproduced levels of the SecYEG mutants were diluted to 1 mg/ml, whereupon the crosslinkers NaTT (1 mM), bBBr (1 mM), BMOE (300 µM) or BMH (600 µM) were added, respectively. The reducing agents DTT and TCEP were used at a final concentration of 30 and 5 mM, respectively. For optimal crosslinking, reactions were performed in a maximum volume of 30 µl and afterwards pooled for analysis. After 30 minutes at 37°C, IMVs were sedimented through a sucrose cushion in 50 mM Tris-HCl, pH 8 and 0.8 M sucrose for 20 min at 80,000 rpm in a TLA 120.1 rotor at 4°C. The pellet was resuspended in 50 mM Tris-HCl pH 8, 20% glycerol and used for further reactions.

An OmpT protease assay was used to assess the crosslinking between cysteine residues in TM2b and TM7 of SecY. Outer membranes containing over-expressed levels of OmpT were diluted to a final concentration of 1 mg/ml in 50 mM Tris-HCl, pH 7, 0.1% Triton X-100. The OmpT solution (7.5 µl) was mixed with the crosslinked IMVs (12 µl) and incubated for 30 min at 37°C. Where indicated, the oxidized cysteine residues were reduced by the addition of an excess DTT (30 mM final concentration), and the samples were separated by 12% SDS-PAGE. SecY was visualized by staining with Coomassie Brilliant Blue R-250 (CBB) or SYPRO® Ruby (Invitrogen) where indicated. Visualization with SYPRO® Ruby staining was done with a Roche Lumi-Imager F1 using a cut-off filter of 600 nm. Western blotting was

performed using antibodies directed against the N-terminal hexa-histidine tag in SecY (Amersham Biosciences).

Crosslinking in the presence of the proOmpA-DHFR translocation intermediate

Folding of the DHFR domain and translocation of the proOmpA-DHFR into IMVs was performed as described [28]. After 30 minutes of translocation at 37°C, the different crosslinkers were added to the reaction mixture and incubated was continued for another 30 min at 37°C. To analyze cross-linking of the cysteine residues, IMVs were treated with OmpT as described above.

Translocation ATPase assay

The SecA ATPase activity during translocation was determined by measuring the amount of released free phosphate using the malachite green assay [158]. Typically, proOmpA (33 μg/ml) was added to translocation buffer containing SecA (20 μg/ml), SecB (400 μg/ml), 1 mM ATP and IMVs (12.5 μg/ml). Pi release was measured after 30 minutes. Measurements were done in triplicate and corrected for background ATPase activity in the absence of proOmpA.

Other techniques

In vitro translocation of proOmpA was performed as described [51]. Typically, fluorescently labeled proOmpA was diluted into translocation buffer containing SecA (20 μg/ml), SecB (400 μg/ml), ATP (1 mM) and IMVs containing

SecYEG derivatives. After 8 minutes at 37°C, the translocation reaction was terminated on ice by treatment with proteinase K, and analyzed by SDS-PAGE and in-gel UV fluorescence using a Roche Lumi-Imager F1 station using cutoff filters of 520 (FL) and 600 nm (TR), respectively. Protein concentrations were determined with the Bio-Rad RC DC protein assay kit using BSA as a standard.

Acknowledgements

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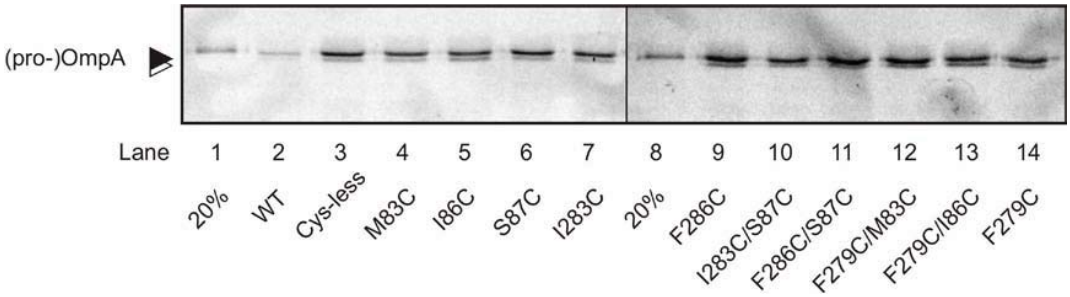

Fig. S1 The SecYEG translocon is active in proOmpA translocation after the introduction of single and double cysteines in TM2b and TM7. FL-proOmpA was diluted into translocation buffer containing SecA (20 µg/ml), SecB (400 µg/ml), ATP (1 mM) and *E. coli* SF100 IMVs containing wild-type levels of SecYEG (WT) (lane 2), overexpressed levels the Cys-less SecYEG complex (lane 3), or bearing the indicated single- (lanes 4-9, 14) or double-cysteine mutations (lanes 10-13). After 8 minutes at 37°C, translocation reactions were terminated on ice by treatment with proteinase K. Samples were precipitated with TCA and protease protected material was analyzed by SDS-PAGE and in-gel UV fluorescence. Lanes 1 and 8 show a 20% FL-proOmpA standard.

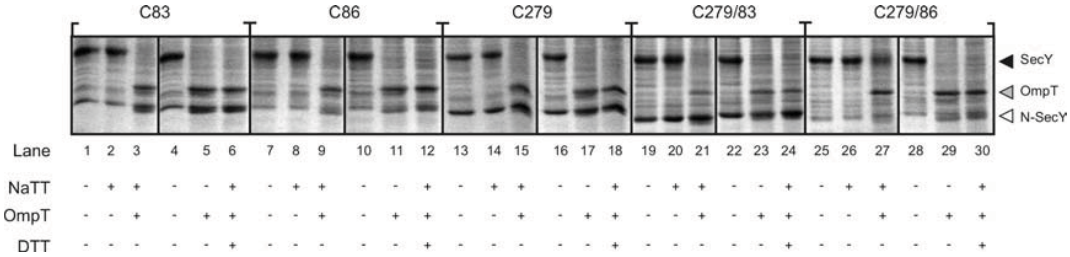

Fig. S2 Disulfide crosslinking of cysteines in the lateral gate formed by TM2b and TM7. IMVs containing different SecYEG derivatives were treated with the oxidizer NaTT (1 mM) as indicated, and incubated with the protease OmpT. Crosslinking of the cysteine residues in SecY was analyzed by SDS-PAGE and CBB staining. In order to prevent the reduction of the disulfide bond during electrophoresis, samples series which contained DTT were analyzed on a separate gel.

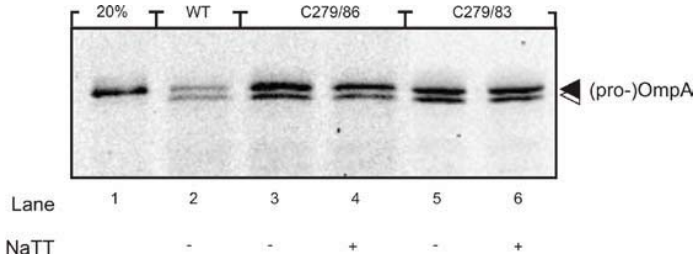

Fig. S3 ProOmpA translocation by IMVs containing the C286/87 and C286/87 SecYEG complexes. IMVs containing the indicated SecYEG derivatives were oxidized or reduced with NaTT (+) and DTT (-), respectively. Translocation of FL-proOmpA was assayed as described in the legend to Fig. S1. Lane 2 shows the translocation by IMVs containing wild-type levels of SecYEG (WT). Lane 1 shows a 20% FL-proOmpA standard.

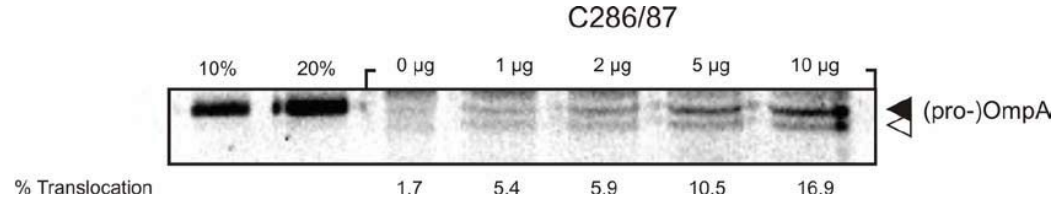

Fig. S4 Limiting amounts of IMVs allow detection of small changes in translocation efficiency. Translocation reaction with TR-proOmpA and the indicated amounts of IMVs containing the SecY 286/87 mutant were performed as described in the legend of Fig. S1. Lanes 1 and 2 show 10 and 20% of TR-proOmpA standards, respectively.

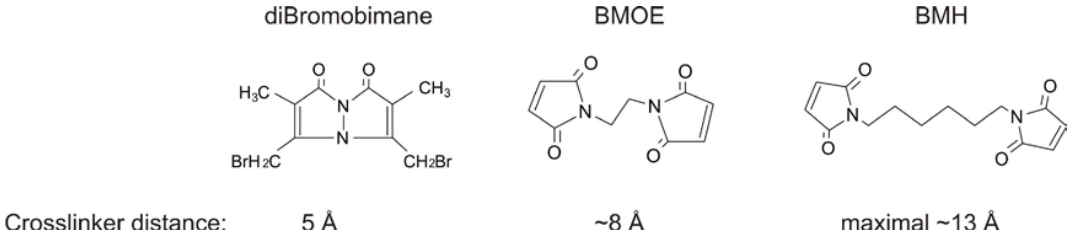

Fig. S5 Chemical structures of crosslinkers used in this study. Dibromobimane (bBBr) is a rigid molecule that reacts covalently with two thiol groups with a spacer distance of 5 Å. bBBr becomes fluorescent when both alkylating groups have reacted with thiols. BMOE (bis-maleimidoethane) is a rigid homo-bifunctional thiol-crosslinker with an 8 Å spacer arm. BMH (bis-maleimido hexane) is a semi-rigid homo-bifunctional thiol-crosslinking reagent with a spacer arm that has a maximal length of 13 Å.

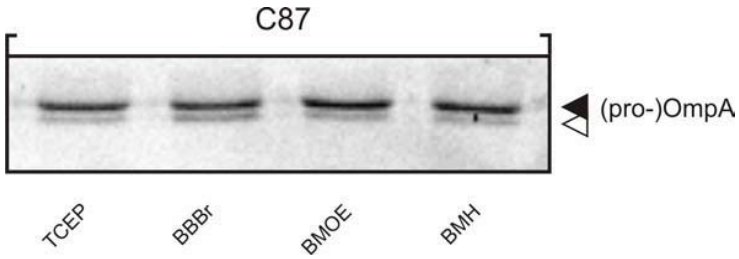

Fig. S6 Crosslinkers with increasing length do not influence the proOmpA translocation activity of the single cysteine SecY mutant C87. IMVs containing single cysteine C87 SecY mutant were reduced with TCEP whereupon samples were treated with different chemical crosslinkers (bBBr, BMOE an BMH) and used in an *in vitro* translocation reaction with TR-proOmpA as substrate. Translocation was assayed as described in the legend to Fig. S1.

Appendix to chapter 3

The lateral gate of SecYEG remains closed during membrane protein insertion

D.J.F. du Plessis, N. Nouwen, A.J.M. Driessen

Introduction

Both the translocation of preproteins across and the insertion of membrane proteins into the cytoplasmic membrane in *Escherichia coli* are mediated by the Sec translocase. While preproteins targeted for secretion contain a signal sequence that is removed upon translocation, most integral membrane proteins lack a signal sequence and instead the first hydrophobic transmembrane segment (TM) functions as a signal for targeting and membrane insertion. About 20 % of the *E. coli* proteome specifies membrane proteins and most of these proteins are targeted as ribosome-bound nascent chains to the translocase by the signal recognition particle (SRP) and the SRP receptor FtsY. Next, the ribosome binds to the SecYEG complex [216] and membrane insertion is coupled to polypeptide chain elongation at the ribosome (co-translational translocation). Importantly, when the membrane protein contains large periplasmic localized polar domains of integral membrane proteins the translocation of these domains requires the activity of the SecA protein. Previous studies suggested that the ribosome and SecA can bind the SecYEG pore simultaneously [313], although the same study showed that SecA can dislodge a ribosome bound to a translocon. The mechanisms by which the activities of these two cytosolic binding partners for SecYEG are coordinated are not resolved, nor is it clear if protein translocation and membrane protein insertion are mediated by the same type of translocase. The ribosome has been proposed to insert the primary TM into the SecYEG channel whereupon the TM, like a signal sequence, may latch in between TM2b and TM7 of the SecY lateral gate. This would result in a widening of the central pore constriction and a subsequent displacement of the periplasmic plug domain and thus the opening of an aqueous pore. Next, adjoining polypeptide segments or TMs would enter the

opened aqueous pore before passively partitioning into the lipid bilayer [107]. Crosslinking studies of multiple membrane-spanning proteins have shown that TMs are released from SecYEG consecutively or in pairs [20]. Whereas recent data demonstrates that opening of the lateral gate is essential for protein translocation [67], its exact role in membrane protein insertion remains poorly understood. Thus far, the only study on its dynamics and role during translocation concerns a molecular dynamics simulation [94] that does not take ribosome binding into account. Here we have investigated the function and the dynamics of the proposed lateral gate located between TM2b and TM7 in membrane protein insertion and lateral release of TMs into the lipid bilayer. It is suggested that co-translationally inserting membrane proteins do not require the opening of the lateral gate, but that lateral gate opening is only needed for SecA mediated translocation.

Results

SecA-dependent membrane insertion of the monotopic and polytopic membrane proteins FtsQ and CyoA requires opening of the lateral gate

In general, transmembrane segments are considerably longer and more hydrophobic than signal sequences. To test the effect of crosslinking of the lateral gate of SecY on the lateral insertion of membrane proteins, we analyzed the insertion of the cytoplasmic membrane protein FtsQ. FtsQ is a 31 kDa monotopic membrane protein (**Fig. 1**A) with a large periplasmic domain (about 22 kDa). FtsQ is co-translationally inserted into the membrane but the translocation of the periplasmic domain is SecA-dependent [265,275,276]. *In vitro*, correct membrane insertion of

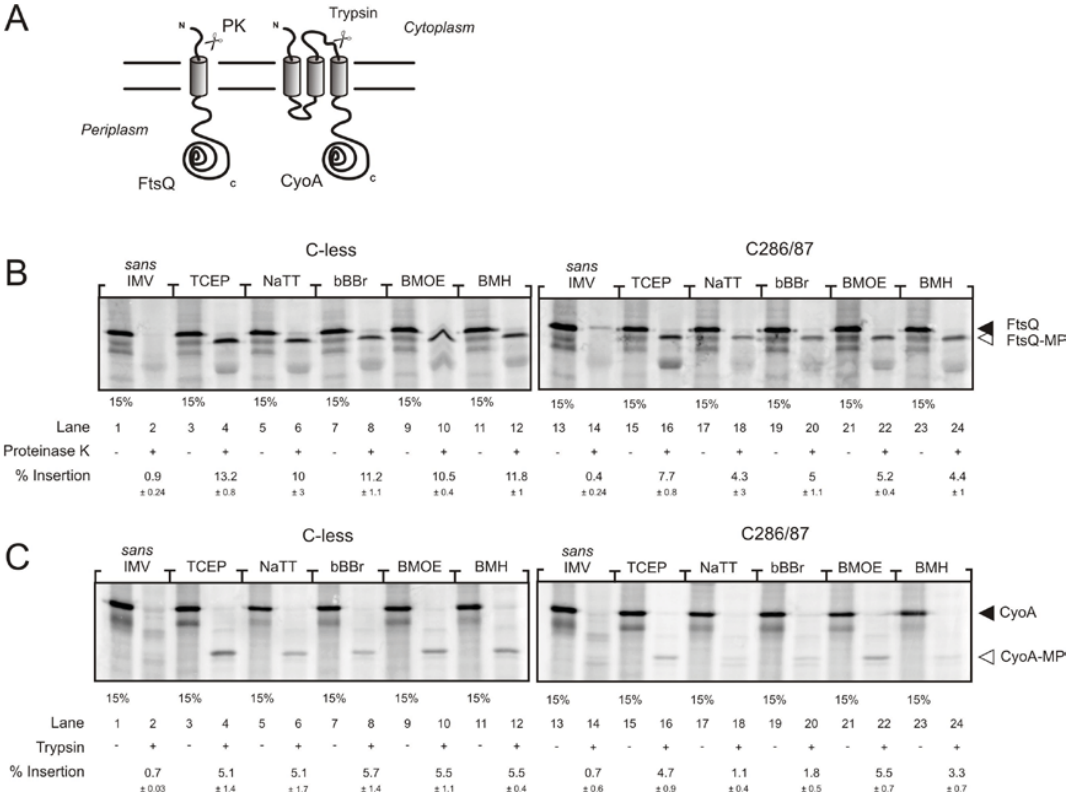

Fig. 1 A disulfide bond and crosslink by bBBr in SecY F286C/S87C blocks SecA-dependent insertion of membrane proteins. (**A**) Schematic representation of the topology of FtsQ and CyoA in the cytoplasmic membrane. ^{35}S-labeled FtsQ and CyoA were synthesized in a DTT-free *in vitro* transcription translation system in the presence of SecY F286C/S87C IMVs that had been treated with TCEP or different chemical cross-linkers. After 30 minutes at 37 °C, trypsin (CyoA, 600 U/ml) or proteinase K (FtsQ, 0,25 mg/ml) was added to degrade non-inserted material. After inactivation of the protease with TCA precipitation, protease protected material was analyzed by 12% SDS-PAGE and autoradiography. (**B**) Membrane insertion of *in vitro* synthesized FtsQ. (**C**) Membrane insertion of *in vitro* synthesized CyoA. 15%, 15 per cent of the total amount of *in vitro* synthesized material. Percentage insertional efficiency of FtsQ and CyoA and corresponding standard deviations were calculated using the 15% synthesis as standard across a minimum of three independent experiments.

FtsQ can be determined using proteinase K that degrades FtsQ when it is not inserted into the membrane while it only cleaves the N-terminal tail of FtsQ yielding a ~27 kDa protease protected fragment (FtsQ-MP) when correctly inserted into the membrane [276]. For membrane insertion, inner membrane vesicles were derived from a strain that overexpresses the cysteine-less SecYEG complex (control) and the SecY(C87/C286)EG mutant that contains unique cysteines in the lateral gate TMS2b and TMS7 [67]. Oxidation of this mutant with sodiumtetrathionate (NaTT) results in the formation of a disulfide bond and this obstructs SecA-mediated protein

translocation. On the other hand, when a cysteine-specific crosslinker (BMOE) is used that introduces a spacing distance between C87 and C286 of 8 angstrom, translocation occur unhindered. FtsQ was synthesized as a ^{35}S-methione labeled protein and co-translationally inserted into the IMVs that had been treated with the oxidizer NaTT, the crosslinkers bBBr, BMOE and BMH or the reductant TCEP. Under all experimental conditions, limiting amounts of IMVs are used to assure that the level of membrane insertion of FtsQ (and also of the other membrane proteins used in this study) is completely dependent on the activity of the overproduced SecYEG [50,276] (See also **Fig. 2**A where wild-type background insertion of FtsQ is indicated). When crosslinking procedure using different crosslinkers was performed with IMVs containing C-less SecYEG this did not affect the subsequent membrane insertion of FtsQ (**Fig. 1**B, C-less, *lanes 1-12*). In contrast, NaTT and bBBr treatment of SecY F286C/S87C IMVs reduced the insertion of FtsQ to levels less than 50 % of the control (**Fig. 1**B, *lanes 18 and 20*) while treatment of the IMVs with the slightly longer crosslinkers BMOE and BMH, only partially inhibited the FtsQ insertion (**Fig. 1**B, compare *lane 22* and *24* vs. *16*). Note, however, that with these larger compounds, the crosslinking of the two cysteine residues is also less efficient (See **Chapter 3**, **Fig. 4**A). Introduction of a "bulky" crosslinker in the lateral gate might hinder the exit of the transmembrane segment from the lateral gate. To determine if the bulkiness of the (BMH) crosslinker influences the insertion of FtsQ for instance we performed FtsQ insertion experiment with SecYEG mutants harbouring the individual cysteines (S87C and F286C, respectively) and that had been modified with BMH. In this case modification of the SecY S87C or F286C IMVs with BMH had no effect on the insertion of FtsQ (**Fig. 2**B). This demonstrates that the decrease in

membrane insertion of FtsQ as found with F286C/S87C IMVs modified with BMH is due to crosslinking of the two cysteines in the lateral gate.

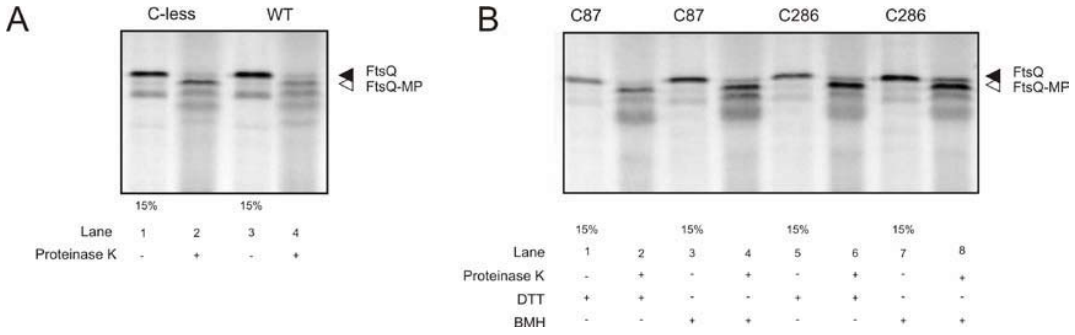

Fig. 2 Insertion of FtsQ into WT and single cysteine mutants. (**A**) Insertion levels of FtsQ into IMVs attributed to wild-type SecYEG. ^{35}S labeled FtsQ was synthesized in a DTT-free *in vitro* transcription translation system in the presence of 5 µg of either cysteine-less (C-less) or wild-type (WT) IMVs. After 30 minutes at 37 °C proteinase K (0,25 mg/ml) was added to degrade non-inserted material. After inactivation of the protease with TCA precipitation, protease protected material was analyzed by 12% SDS-PAGE and autoradiography. (**B**) Presence of the long and flexible crosslinker BMH on either S87C (TM2) or F286C (TM7) did not hinder the membrane insertion of FtsQ. FtsQ was synthesized in a DTT-free *in vitro* transcription translation system in the presence of 5 µg of IMVs harbouring over expressed levels of either S87C and F286C SecY.

CyoA (**Fig. 1**A) is the 32 kDa subunit of the cytochrome bo_3 ubiquinol oxidase that originates from a precursor whose signal peptide is cleaved by leader peptidase II [162]. Mature CyoA consists of two transmembrane segments and a large periplasmic domain that is translocated across the membrane [2]. The co-translational insertion of CyoA into the cytoplasmic membrane of *E. coli* is dependent on SecYEG, SecA as well as YidC and does not require the PMF [68]. Membrane insertion of CyoA can be reconstituted *in vitro* using a transcription-translation system. Correct insertion of ^{35}S-methione labeled CyoA can be verified by trypsin treatment which results in a 25 kDa protease protected fragment corresponding to the translocated large periplasmic domain [68]. Notably, efficient insertion requires overproduced levels of SecYEG in IMVs, where wild-type vesicles show only low levels of insertion [68]. Membrane insertion of CyoA into IMVs containing the Cys-less SecYEG and treated with the different chemical crosslinkers was only slightly lower

as compared to the non-treated IMVs **(Fig. 1**C). In contrast, CyoA insertion was completely abolished when SecYEG F286C/S87C IMVs were used that were treated with the oxidizer NaTT (**Fig. 1**C *lane 18* and *14*, respectively). Insertion of CyoA into bBBr-treated IMVs was also strongly reduced (Compare **Fig. 1**C, *lanes 20* and *18*). Remarkably CyoA insertion into BMOE modified SecYEG F286C/S87C IMVs, occurred at almost similar levels as observed with reduced SecYEG F286C/S87C IMVs and the Cys-less IMVs modified with BMOE (**Fig. 1**C, *lane 22*). Moreover, insertion of CyoA was also inhibited when SecYEG F286C/S87C IMVs were used that were treated with the longer crosslinker BMH (**Fig. 1**C, *lane 24*) whereas these IMVs are highly active for proOmpA translocation [67]. Overall, these data indicate that the membrane insertion of FtsQ and CyoA require a lateral gate opening that is similar to proOmpA, i.e., a highly constrained lateral gate (with NaTT and bBBr) inhibits membrane insertion, while insertion can take place in IMVs that are treated with a slightly larger crosslinker BMOE. However, in contrast to proOmpA translocation, further widening of the lateral gate due to crosslinking of the cysteine residues with the longer crosslinker BMH strongly interferes with the membrane insertion of the polytopic membrane protein CyoA.

The SecA-independent membrane insertion of the polytopic membrane protein MtlA does not require lateral gate opening

Both FtsQ and CyoA are membrane proteins with a large periplasmic domain that requires SecA for translocation across the cytoplasmic membrane [68,276]. For this reason, we decided to analyze the effect of a constrained lateral gate using a membrane protein that does not depend on SecA for insertion. Mannitol permease

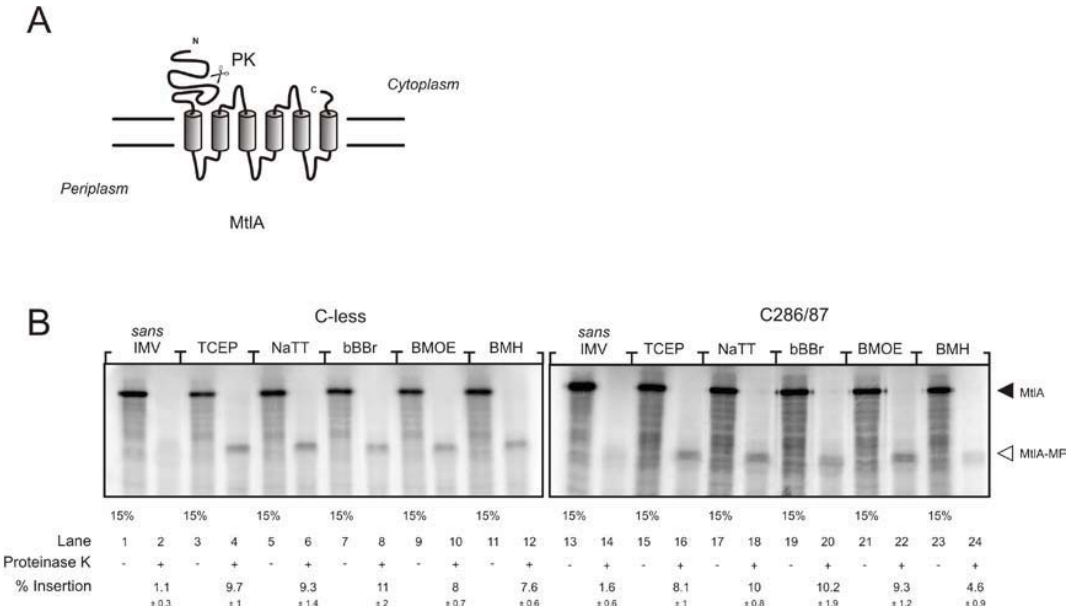

Fig. 3 A disulfide bond and crosslink by bBBr in SecY F286C/S87C does not block membrane insertion of the polytopic SecA-independent membrane protein MtlA. (**A**) Schematic representation of the topology of MtlA in the cytoplasmic membrane. (**B**) ^{35}S-labeled MtlA was synthesized in a DTT-free *in vitro* transcription translation system and inserted into different treated SecY F286C/S87C IMVs as described above. Proteinase K protected material was analyzed by 15% SDS PAGE and autoradiography. 15%, 15 per cent of the total amount of *in vitro* synthesized material. Next to the gel the position of the proteinase K resistant membrane domain of MtlA (MtlA-MP) is indicated. Percentage insertional efficiency of MtlA and corresponding standard deviations were calculated using the 15% synthesis as standard across a minimum of three independent experiments.

(MtlA) is a 68 kDa polytopic membrane protein with 6 TMs and no large periplasmic domains (**Fig. 3**A). It neither require SecA nor SecB for its insertion, but inserts co-translationally in a SecYEG-dependent manner [296]. MtlA shows significantly higher levels of insertion with IMVs containing overexpressed levels of SecYEG when compared to wild-type levels [50]. Correct membrane insertion of *in vitro* synthesized ^{35}S-methionine labeled MtlA results in the protease protection of the membrane integrated fragment (30 kDa), while the cytosolic phosphorylation domain is proteolyzed [50,296]. Treatment of IMVs containing the C-less SecYEG with the various crosslinkers did not affect the membrane insertion of MtlA (**Fig. 3**B, *lanes 1-12*). Remarkably, MtlA inserts normally into SecY F286C/S87C IMVs treated with the NaTT or the short crosslinker bBBr up to levels similar to the TCEP treated vesicles (**Fig. 3**B, *lanes 18* and *20* versus *lane 16*). This strongly suggests that

membrane insertion of MtlA does not require the opening of the lateral gate. Remarkably, similar to the membrane insertion of CyoA, treatment of SecY F286C/S87C IMVs with the flexible 13 Å crosslinker BMH dramatically inhibited MtlA insertion (**Fig. 3**B, *lane 24*). Possibly because of the bulkiness of the BMH crosslinker, the lateral gate cannot close and insertion of hydrophobic TMs is inhibited.

Discussion

In this study, we have addressed the role of the lateral gate in the insertion of membrane proteins into the cytoplasmic membrane via SecYEG. In *E. coli*, membrane protein insertion by the translocon is a co-translational event that is linked to polypeptide chain elongation at the ribosome. Here, we have studied two modes of membrane protein insertion via the Sec translocon. First, membrane proteins such as FtsQ that contain a large periplasmic domain and a single TMS, and that in addition require SecA for correct insertion. Likewise, subunit a of cytochrome *o* oxidase, CyoA. However, CyoA differs from FtsQ in that it contains multiple TMS. Second, the polytopic membrane protein MtlA that lacks large polar loops and that inserts independently of SecA. In analogy to proOmpA translocation, membrane insertion of both FtsQ and CyoA is hindered when the lateral gate is fixed by oxidation (NaTT) or by the short crosslinker (bBBr), while insertion occurs largely at unrestricted levels when a longer crosslinker (BMOE) is introduced in the lateral gate (**Figs. 1**B and **1**C). Apparently, complete membrane insertion of these proteins requires the opening of the lateral gate, as observed for SecA-mediated proOmpA translocation. Previous studies with nascent FtsQ showed that the N-terminal TM segment can be crosslinked

to SecY independent of the presence of SecA [265] suggesting that SecA is recruited to the translocon only at a later stage. Thus, the translocon may switch between different translocation modes. This notion is supported by the differential behavior of the R357 mutations in SecY that interferes with SecA-dependent (post-translational) initiation of preprotein translocation, but that normally allow for ribosome (co-translational) dependent initiation of membrane protein insertion [50]. We therefore hypothesize that in case of FtsQ and CyoA the initial membrane insertion of the N-terminal TM segment(s) occurs without lateral gate opening, while lateral gate opening is obligatory for the SecA-dependent translocation of the periplasmic domain. This hypothesis is supported by the observation that except for BMH, membrane insertion of MtlA occurs largely unrestricted when the lateral gate of SecY is constrained by chemical crosslinking. These data demonstrate that a closed lateral gate does not affect the co-translational insertion of membrane proteins such as MtlA that do not require SecA for assembly. Apparently, TMSs can leave the translocon without the need for the lateral gate opening at TM2b and TM7. Based on the three available structures of SecY, one possible route by which TM segments might exit the translocon without the need of completely entering the aqueous pore is almost horizontally through the lateral gate region over the angled TM2b and TM7 into the lipid bilayer. However, this suggestion should be tested in future experiments.

Remarkably, with the longer yet more flexible 13 Å crosslinker BMH, proOmpA translocation occurred normally (**Chapter 3**) while the insertion of the membrane proteins CyoA and MtlA was severely affected. One possible explanation for this behavior is that the bulkiness of BMH prevents closure of the lateral gate and this may result in a stabilization of the open vectorial pore state which seems incompatible with membrane protein insertion. The open state of the SecY pore might

be unable to interact functionally with the ribosome or alternatively, the insertions of the newly synthesized TMS into an open aqueous SecY pore is thermodynamically unfavorable and insertion is inhibited.

In conclusion, in contrast to protein translocation (**Chapter 3**) the insertion of TM segments does not seem to require the opening of the TM2b/TM7 lateral gate. As the crosslinkers stabilize the lateral gate at the position of the plug domain, displacement of the plug might not occur during TMs integration. As a model for TM integration we hypothesize that hydrophobic TM sequences slide into the membrane at the TM2b/TM7 interface at the cytosolic face of the membrane, rather than inserting first in a vectorial manner into the aqueous gate region followed by subsequent lateral diffusion into the lipid bilayer. Signal sequences, however, may leave the translocon via the TM2b and TM7 gate as the opening of the lateral gate and activation of the SecA ATPase activity are tightly interlinked events. Taken together, the SecYEG translocon seems to be a flexible structure that undergoes specific conformational changes depending on the cytosolic factor that it binds, i.e., SecA or ribosomes.

Experimental procedures

Chemicals and biochemicals

In vitro transcription-translation-insertion was performed using ^{35}S-labeled Promix (Amersham Pharmacia Biosciences) [271]. For all experiments involving either preprotein translocation or insertion of membrane proteins the same batch of reduced and crosslinked IMVs were used.

Bacterial strains and plasmids

The following two plasmids were utilized for the coupled *in vitro* transcription-translation-insertion reaction. Plasmid pBSKftsq for the *in vitro* synthesis of FtsQ [276] and plasmid pET27bCyoA for the *in vitro* synthesis of CyoA [68].

In vitro transcription-translation-insertion

As disulfide bonds are easily reduced, an *in vitro* transcription-translation system was developed devoid of reducing agents. A cell lysate was prepared by inoculating 50 ml double strength (2x) YPTG media [135] with a single colony of BL21 (DE3) Rosetta (Novagen). After overnight incubation at 37 ºC (250 rpm) the pre-culture was used to inoculate a 4 L Erlenmeyer containing 1 L of 2x YPTG media [135]. When the OD_{600} reached a value of 0.6, the culture was chilled in an ice-water bath. Cells were harvested by centrifugation (15 min at 7,500 rpm JLA 10-500 rotor; 4 ºC). Cells were washed with buffer A (10 mM Tris-acetate, pH 8.0, 14 mM $Mg(OAc)_2$, 60 mM KCl, and 50 µg/ml PMSF) thereafter the cells were suspended in 2 ml buffer A per gram of cells and lysed by passage through a cell disruptor (Constant Cell Disruption Systems (UK); 10,000 psi). Cell debris was removed by centrifuging (10,000 rpm SS34 rotor (Beckman); 4 ºC). The supernatant was transferred to an MLA-80 tube and centrifuged for 30 minutes at 25,000 rpm in a MLA-80 rotor (4 ºC). The supernatant was transferred to a 5 ml falcon tube, supplemented with 55 mM sodium pyruvate, 45 µM coenzyme A and 110 µM nicotinamide adenine dinucleotide

(NAD). After 90 minutes in a water bath (37 ºC) in the dark, membranes were removed by ultracentrifugation (30 minutes 52,000 rpm MLA-80 rotor, 4 °C). The cell lysate was dialyzed for 24 hours against 1 liter of buffer A *sans* PMSF (4 ºC, molecular weight cut-off of 6,000 to 8,000 dalton). In this period, the buffer was replaced three times. After dialysis, the lysate was aliquoted, snap-freezed in liquid nitrogen and stored at -80 ºC. DTT-free *in vitro* transcription-translation reactions were performed as described [120] using T7 polymerase from Fermentas and addition of ^{35}S-methionine (Amersham Pharmacia Biosciences) and 5 µg of crosslinked IMVs to the reaction mix [271].

Translocation and membrane protein insertion occur unhindered via front-to-front crosslinked SecYEG translocons

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In submission (2010)

Summary

The SecYEG translocase of *E. coli* mediates the translocation of proteins across, and the insertion of membrane proteins into, the cytoplasmic membrane. Biochemical and structural data indicate the presence of dimers in addition to the monomeric SecYEG complex. The orientation and functional significance of the SecYEG dimer is unknown. Here we have employed site-specific cysteine mutagenesis to stabilize the SecYEG dimer in a front-to-front orientation while precluding the formation of a consolidated pore. In the crosslinked state, protein translocation and membrane protein insertion remains equally functional as compared to non-crosslinked translocons. These data suggest that the front-to-front dimeric arrangement of the translocase is completely functional and indicate a high plasticity of the SecYEG interacting surface during dimerization.

Introduction

In *Escherichia,* translocation of preproteins across and the insertion of membrane proteins into the inner membrane are mediated by the Sec translocase. The translocase consists of a heterotrimeric SecYEG integral membrane protein complex that either associates with the ATPase motor protein SecA or the ribosome (For a recent review see [65]). The structure of the unliganded SecYEβ complex of the archaeon *Methanocaldococcus jannaschii* has been solved [270]. SecY, the largest of the subunits in this complex, consist of an N-terminal and C-terminal domain that are arranged in a clamshell structure comprising the transmembrane segments (TMs) 1-5 and 6-10, respectively. This structure encompasses an hourglass shaped central pore, through which protein translocation is thought to occur. The central pore is closed at the periplasmic face of the membrane by a small plug-like domain formed by TM2a. The SecE protein embraces the clamshell structure in a vice-like manner forming the "back" of the complex. The "front" of the complex has been proposed to constitute a lateral gate positioned between TM2b and TM7. When opened, it will expose the central aqueous pore to the lipid phase of the membrane. Lateral gate opening may be inflicted by the signal sequence or by TM segments that are subsequently released into the lipid phase [270]. Recent evidence indicates that lateral gate opens during protein translocation and that this process is allosterically coupled to the activation of the SecA ATPase [67]. Two additional structures of bacterial translocons have been reported [259,311] and these highlight the remarkable structural and functional conservation between translocons of different domains of life.

The crystal structures of all translocons are monomeric. However, various lines of biochemical evidence indicate that the protein complex is able to form higher

oligomeric states. Notably, such evidence was provided by crosslinking experiments [131,164,235,252,279], electron microscopy and fluorescence energy transfer [235]; 2D crystallography of the unliganded translocon and the translocon associated with the ribosome [32,44,171,262], analytical ultracentrifugation [44] and blue-native gel electrophoresis [24]. On the other hand, the functional significance of the various oligomeric states have remained unclear [24,164,201,235,262,303,311]. Although structural studies on the *Thermotoga maritima* SecA-SecYEG complex suggest that a monomeric arrangement of the complex may suffice for protein translocation [78], a directed crosslinking approach suggests a functional asymmetry in a dimeric translocon wherein one translocon provides a binding site for the SecA motor protein while the other translocon would form the actual translocation pore [201]. In the latter dimeric arrangement, only one of the pores is active in protein translocation. Based on the proposed dimeric structure of the SecYEG complex bound to a translating ribosome, it was also suggested that one of the pores functions in protein translocation while the other would be involved in the release of hydrophobic TMs into the lipid bilayer [171]. Although the orientation of the SecYEG complexes within the dimer is unknown, the position of the linker used to fuse two SecY proteins suggests a so-called 'back-to-back' orientation [32,131,286]. This dimeric translocon will accommodate two separate pores. In the other proposed 'front-to-front' orientation [169,171] the two lateral gates face each other and when properly aligned there is the possibility that the pores fuse to yield a single consolidated channel. Through the use of cysteine mutagenesis experiments, we have previously shown that a residue in the transmembrane segment (TM) of SecE that is nearby the corresponding TM of another monomeric translocon can be utilized to create a back-to-back orientation of two translocons [131,286]. However, this SecE disulfide crosslink reversibly

inactivated protein translocation. Moreover, using the back-to-back SecE crosslink as diagnostic marker of the dimer orientation, no disulfide bond could be formed under conditions of translocation initiation. These data suggest that the SecYEG dimer is a highly dynamic entity possibly re-arranging or even dissociating during translocation initiation.

Here we have employed site-specific cysteine mutagenesis to stabilize the *E. coli* translocon in a dimeric front-to-front orientation at the lateral gate region. In this state, the formation of a consolidated channel is precluded by the presence of the disulfide crosslink at the periplasmic face of the membrane. The data demonstrate that a stably front-to-front crosslinked translocon is functional both in preprotein translocation and membrane protein insertion.

Results

Introduction of cysteines into the SecYEG translocon to facilitate dimeric crosslinking

To investigate the role of a front-to-front dimeric state of SecYEG in protein translocation, we introduced single cysteine point mutations into a cysteine-less SecYEG translocon (**Fig. 1**A). To select amino acid positions that might be in close proximity in the putative front-to-front dimer, potential amino acid residues in the lateral gate region were selected in the *E. coli* SecYEG based on homology with the *M. jannaschii* SecYEβ structure [270]. Unique cysteines were introduced at positions L148 and G299 that are at the periplasmic ends of transmembrane segments (TM) 4 and 7, respectively (**Fig. 1**A). In the proposed front-to-front SecYEG dimer, the cysteine in the first translocon will crosslink with the corresponding cysteine position

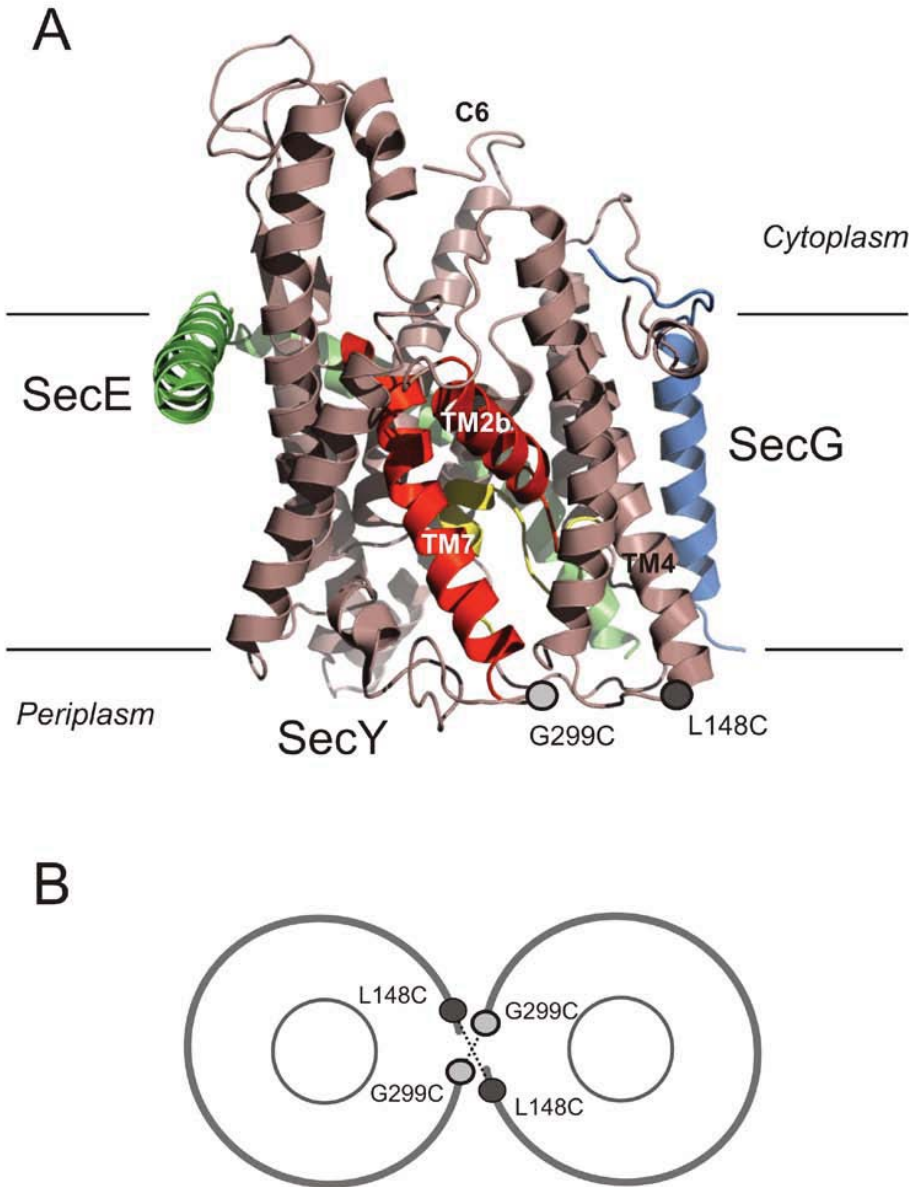

Fig. 1 Design of front-to-front crosslinked SecYEG constructs. (**A**) Side view of the *M. jannaschii* SecYEβ crystal structure. Indicated are the relative positions for the introduced single cysteines in a cysteine-less SecY background. SecY is depicted in salmon red, with SecE and β-subunit depicted in green and light blue, respectively. The lateral gate formed by TM7 and TM2b is highlighted in deep red, with the central plug domain (TM2a) highlighted in yellow. Single cysteine mutants introduced are L148C (light grey) on TM4 and G299C (dark grey) on TM7. The structural view was obtained using PyMol using the coordinates 1RZH.pdb. (**B**) Schematic representation of two *E. coli* SecY translocons in a front-to-front orientation as viewed from the cytoplasmic side. The relative positions of the various single cysteine mutations are indicated as grey dots.

in the opposing translocon. As a consequence, the disulfide crosslink will prevent a proper alignment of the two lateral gates and thus obstruct the formation of a single consolidated channel (**Fig. 1**B). The previously described back-to-back translocon

crosslinked via SecE [131,286] was shown to be inactive in protein translocation upon crosslinking and was therefore not used in this study.

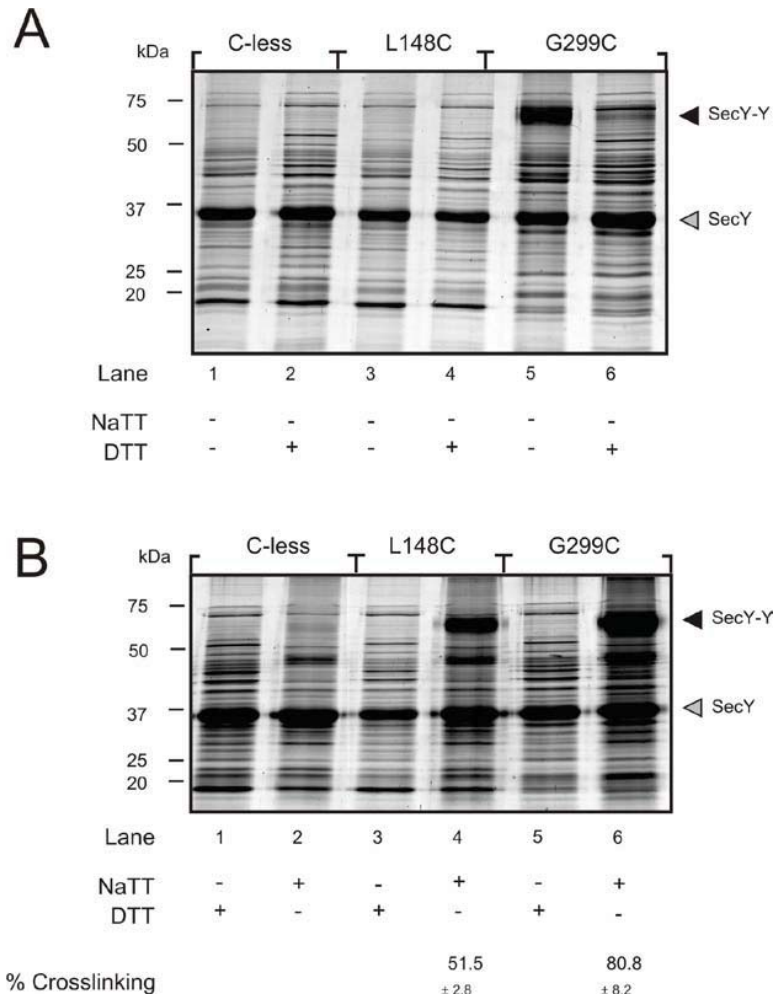

Fig. 2 Single cysteine SecY mutants L148C and G299C are forming dimers upon oxidation with sodium-tetrathionate (NaTT). (**A**) SecY G299C spontaneously form dimers in a reductant-free environment. Coomassie stained SDS-PAGE gel of IMVs isolated in a reductant-free environment, harboring the indicated single cysteine SecY mutations. (**B**) Single cysteine SecY mutants L148C and G299C form dimers in the presence of 5 mM NaTT as oxidizing agent. Coomassie stained SDS-PAGE gel of vesicles treated with either 5 mM NaTT or 20 mM dithiothreitol (DTT). Indicated by arrows are the position of SecY and the SecY-Y dimer and the efficiency of crosslinking.

After overproduction of the various single cysteine mutants in *E. coli* and subsequent isolation of the inner membrane vesicles (IMVs) in a reductant-free environment, we found that the single cysteine mutant G299C had a high propensity to spontaneously oxidize even without the addition of a chemical crosslinking reagent

(**Fig. 2**A, *lane 5*). After treatment with 20 mM dithiothreitol (DTT) the SecY-Y dimer dissociated into non-crosslinked SecY monomer as shown by SDS-PAGE (**Fig. 2**A, *lane 6*). Upon addition of the oxidizing reagent sodium-tetrathionate (NaTT) both single cysteine mutants formed SecY-Y dimers (**Fig. 2**B *lanes 4* and *6*), whereas no SecY-Y dimers were formed with IMVs containing cysteine-less (Cys-less) SecY (**Fig. 2**A and 2B, *lane 2*). Quantification of the amount dimeric and monomeric SecY showed that the G299C mutant was crosslinked at high efficiency of 81%, where the L148C mutant exhibited a maximum crosslinking efficiency of only 52%.

Translocation of proOmpA is unaffected by a front-front crosslinked state of the translocon

To determine if the mutations affect the translocation activity of SecYEG, we analyzed the *in vitro* translocation of fluorescein labeled proOmpA (FL-proOmpA) into IMVs containing the different SecYEG mutants under reducing conditions. To ensure that the activity of SecYEG is rate determining in the *in vitro* translocation reactions, we first performed a translocation reaction with increasing amounts of IMVs where the increase in proOmpA translocation is linear up to ~ 2 µg IMVs in the translocation assay (data not shown). In the remainder of this study, this amount (2 µg) of IMVs was used. Under reducing conditions, IMVs containing the single cysteine mutations at positions 148 and 299 translocated proOmpA with the same efficiency as IMVs containing Cys-less SecYEG (**Fig. 3**A). The same results were obtained when IMVs were used that had been oxidized with 5 mM NaTT to yield the disulfide-bonded dimers (**Fig. 3**B). These data indicate that a disulfide-bonded front-to-front SecYEG dimer is functional in protein translocation.

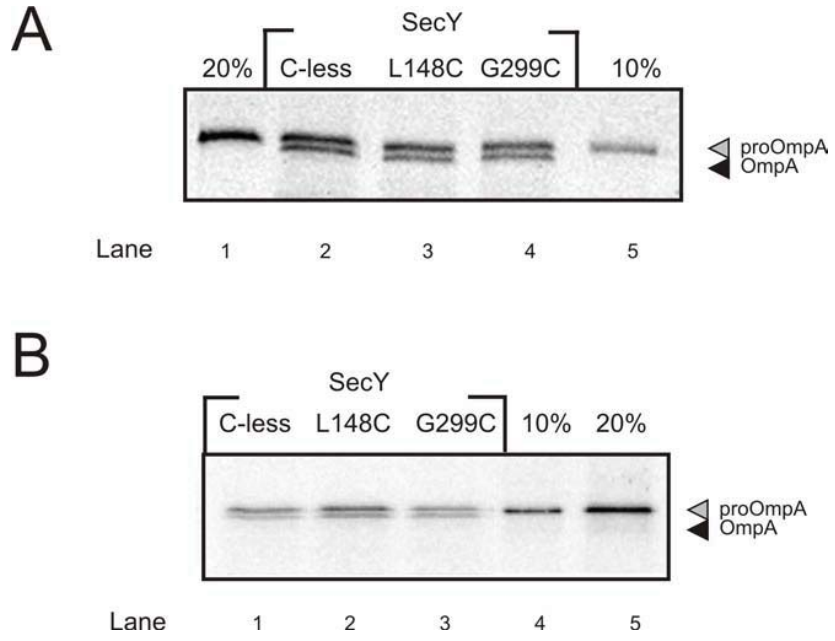

Fig. 3 Oxidation of SecY in a stable front-to-front SecY dimer does not interfere with proOmpA translocation. Fluorescein labeled proOmpA was diluted into translocation buffer containing SecA, (20 μg/ml) SecB (400 μg/ml), ATP (1 mM) and 2 μg IMVs containing SecYEG derivatives that had been oxidized or reduced with NaTT and DTT, respectively. After 8 minutes at 37 °C, the translocation reaction was terminated on ice by treatment with proteinase K. Samples were precipitated with TCA and protease protected material was analyzed by SDS-PAGE and in-gel UV fluorescence. (**A**) IMVs containing the single cysteine SecY mutants L148C and G299C translocate fluorescein-labeled proOmpA (C245) in the presence of DTT. (**B**) NaTT treated IMVs containing single cysteine SecY mutants L148C and G299C translocate fluorescein-labeled proOmpA. Standards of 10 and 20% of the total amount proOmpA used in a translocation reaction are indicated.

Crosslinked SecY front-to-front dimers are fully functional with a covalently-linked SecA dimer

In the above experiments an excess amount of SecA was used. To determine if the disulfide-bond stabilized SecYEG dimers normally interact with the SecA motor protein, we analyzed the SecA-dependence of translocation. As the G299C mutant exhibited the highest crosslinking efficiency, with little uncrosslinked SecYEG, the SecA dependence of proOmpA translocation of this mutant was analyzed. It is expected that when a stable SecY-Y dimer can only accommodate a monomeric SecA molecule, that the crosslinked SecYEG complex will saturate at a two-fold lower SecA concentration as compared to a noncrosslinked complex. Experiments with

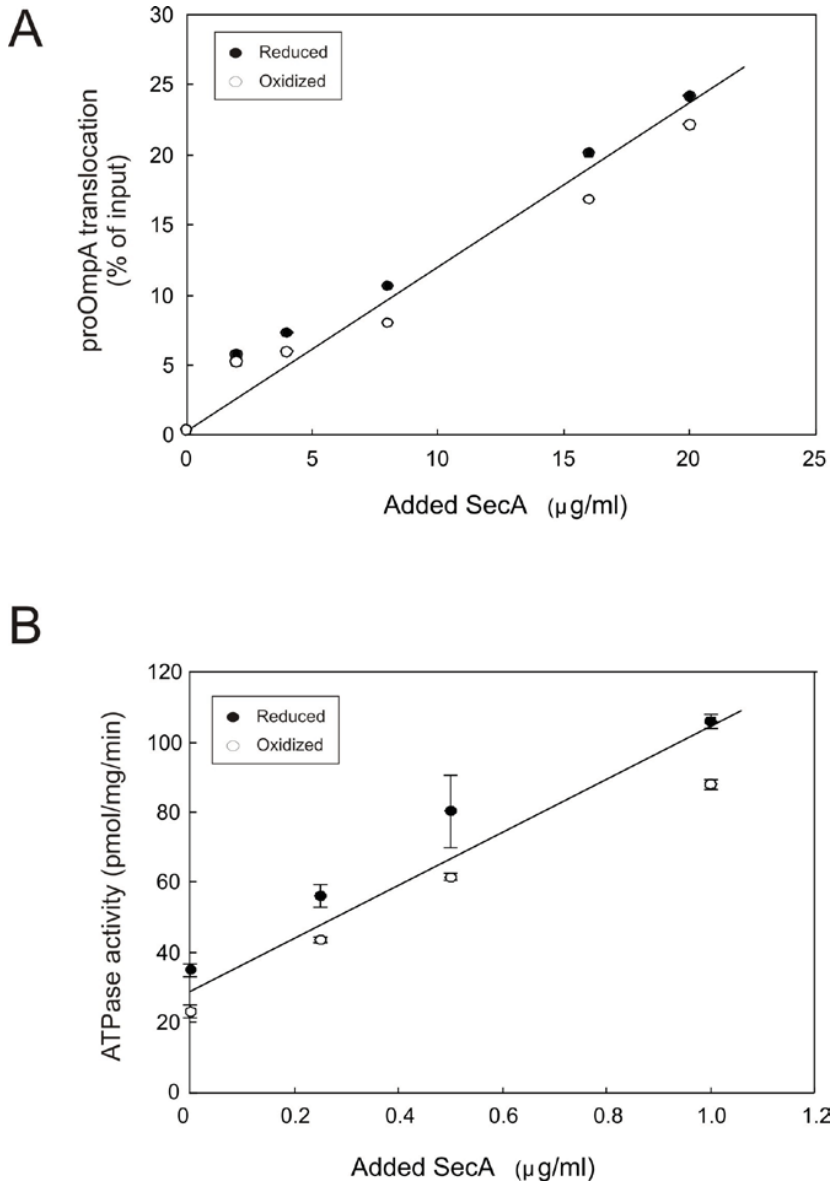

Fig. 4 Oxidation of SecYEG into a stable front-to-front dimer does not affect the SecA dependence of proOmpA translocation and proOmpA stimulated SecA ATPase activity. (**A**) Translocation of fluorescein-labeled proOmpA into IMVs containing SecY(G299C)EG under reducing (black) or oxidizing (white) conditions and increasing SecA concentrations. The reaction was initialized with the addition of the precursor. (**B**) proOmpA stimulated ATPase activity with increasing SecA concentrations in the presence of urea stripped IMVs containing SecY(G299C) under reducing (black) or oxidizing (white) conditions. The reaction was initialized with the addition of SecA. In both the proOmpA translocation and SecA ATPase activity experiments, the final SecA to IMV ratio was 1:5.

limiting amounts of SecA showed that the SecA dependence of FL-proOmpA translocation into IMVs harboring the reduced (black) or oxidized (white) G299C translocon is the same (**Fig. 4**A). Similarly, the SecA dependence of the proOmpA

stimulated SecA ATPase activity with the SecY G299C mutant in its reduced (black) and oxidized (white) state was similar (**Fig. 4**B). Due to the high sensitivity of the colorimetric assay in determining the ATPase activity (**Fig. 4**B), the amounts of both IMVs and SecA were reduced to ensure the linearity of the assay. However, the concentration ratio between SecA and the IMVs remains identical in both the translocation and ATPase experiments. These results indicate that the crosslinked front-to-front SecYEG translocon is not hindered in its ability to functionally interact with the SecA motor protein.

Next, we determined the oligomeric state of SecA that associates with the front-to-front crosslinked translocons. Previously, we have found that the oxidation of SecA into dimers did not affect the SecB-independent translocation of proOmpA and the proOmpA-stimulated SecA ATPase activity [53]. Stable dimerization of SecA is readily achieved by crosslinking the naturally occurring cysteine residues in the carboxyl-terminus of SecA with the same positions on another SecA. The carboxyl-terminus of the *E. coli* SecA comprises 3 cysteine residues which are essential for Zn^{2+} ion binding and SecB interaction [80,309]. Upon removal of the Zn^{2+} ion by EDTA treatment, the wild-type SecA was almost completely converted into a crosslinked dimer using copper-phenanthroline as oxidizing agent. Crosslinking of SecA at room temperature was much more efficient as compared to 4 °C (**Fig. 5**A, *lane 4* vs *lane 6*). Treatment of the dimeric SecA with the reductant DTT reversed the crosslinking yielding monomers on SDS-PAGE (**Fig. 5**A, *lanes 3* and *5*). Translocation of FL-proOmpA into IMVs containing Cys-less SecY by the native SecA (**Fig. 5**B, *lane 3*) or the crosslinked SecA dimer (**Fig. 5**B, *lane 9*) was similar. Importantly, addition of DTT to reverse the crosslinking of the SecA dimer did not result in a more efficient translocation of proOmpA (**Fig. 5**B, compare *lanes 9* and *10*

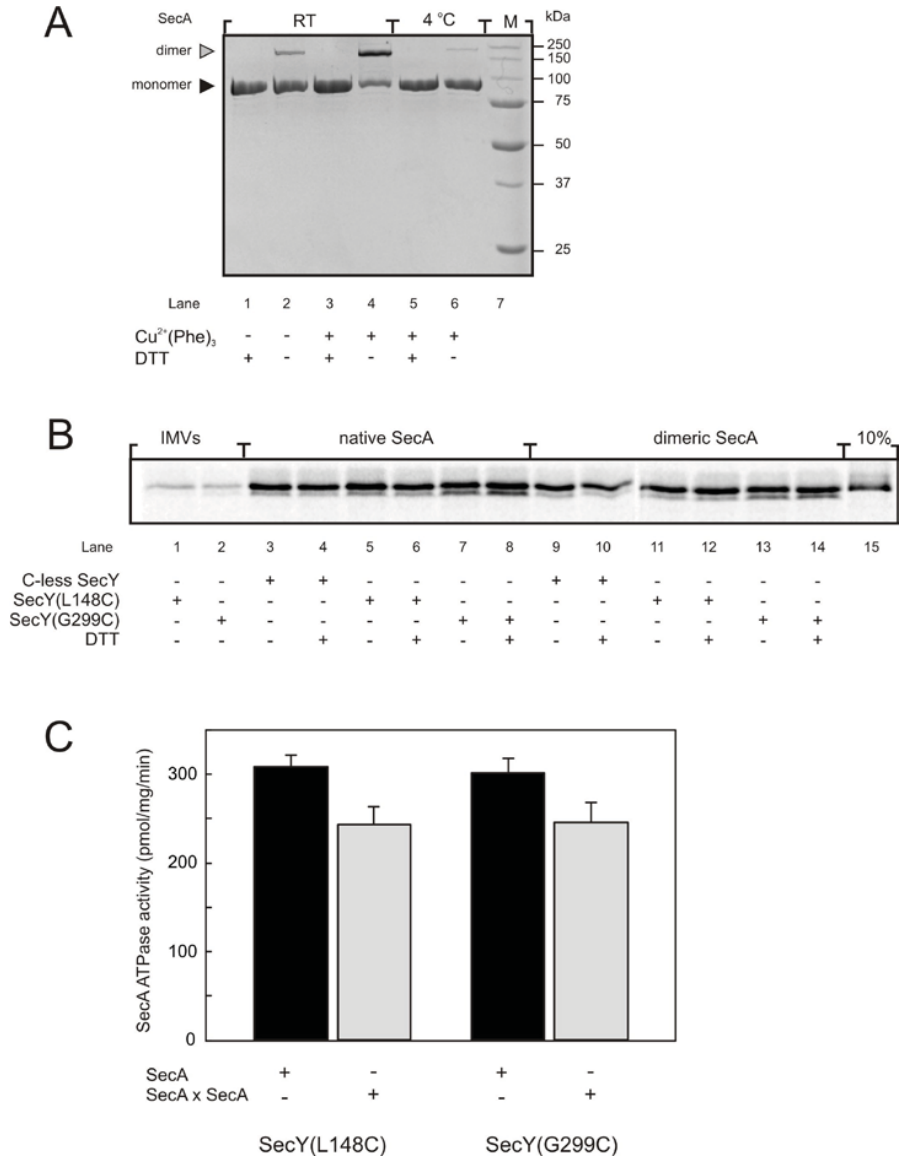

Fig. 5 Crosslinked dimeric SecA is active on front-to-front crosslinked dimeric translocons. (**A**) Purified SecA was incubated with 2 mM EDTA and 1 mM $Cu^{2+}(Phe)_3$ at room temperature (RT) and at 4 ^{o}C to facilitate dimerisation of SecA. Samples were analyzed on a non-reducing Coomassie stained SDS-PAGE gel. (**B**) Translocation of fluorescein labeled proOmpA into IMVs containing overexpressed levels of the Cys-less SecYEG and NaTT treated L148C or G299C SecYEG translocons. For each type of IMV, translocation reactions were performed with either reduced or oxidized SecA. (**C**) ATPase activity of SecA in the presence of IMVs containing NaTT treated L148C or G299C mutants. Black bars represent the ATPase activity of reduced SecA in the presence of a dimeric translocon while the grey bars represent the ATPase activity of oxidized SecA in the presence of a dimeric translocon.

for crosslinked SecA, and *lanes 3* and *4* for the native SecA). These observations confirm previous results with the crosslinked SecA utilizing native translocons [53]. Interestingly, when the oxidized SecA was used in translocation reactions containing

IMVs with the dimerized front-to-front translocons, again proOmpA was translocated normally (**Fig. 5**B, *lane 11* and *13*) to an identical level as the native SecA (**Fig. 5**B, *lane 5* and *lane 7*). Again, as a control DTT was added to the crosslinked SecA and this did not further stimulate translocation (**Fig. 5**B, *lane 12* and *14*). Likewise, the proOmpA-stimulated ATPase activity of the oxidized dimeric SecA (**Fig. 5**C, grey bars) and native SecA (**Fig. 5**C, black bars) in the presence of front-to-front crosslinked L148C (**Fig. 5**C, *lane 2* vs *lane 1*) and G299C (**Fig. 5**C, *lane 4* vs *lane 3*) SecY mutants was very similar. These data indicate that a dimeric SecA motor protein is able to bind in a native manner to the front-to-front SecYEG dimer with no significant loss in ATPase and translocation activity.

Membrane insertion of FtsQ and MtlA is not affected by front-to-front crosslinking of translocons

Transmembrane segments of membrane proteins that insert via the SecYEG translocon are thought to leave the translocon via a lateral gate constituted by TM2b and TM7 of SecY [270]. Since the lateral gate is at the interface of the front-to-front SecYEG dimer, the effect of crosslinking on the insertion of two distinct membrane proteins was analyzed.

FtsQ is a 31 kDa monotopic membrane protein (**Fig. 6**A) with a large periplasmic domain of 22 kDa that is co-translationally inserted into the membrane in a SecA-dependent manner [265,275,276]. *In vitro*, correct membrane insertion of FtsQ is determined using proteinase K that degrades FtsQ when it is not inserted into the membrane and only cleaves the N-terminal tail of membrane inserted FtsQ yielding a ~27 kDa protease protected fragment (FtsQ-MP) [276]. FtsQ was

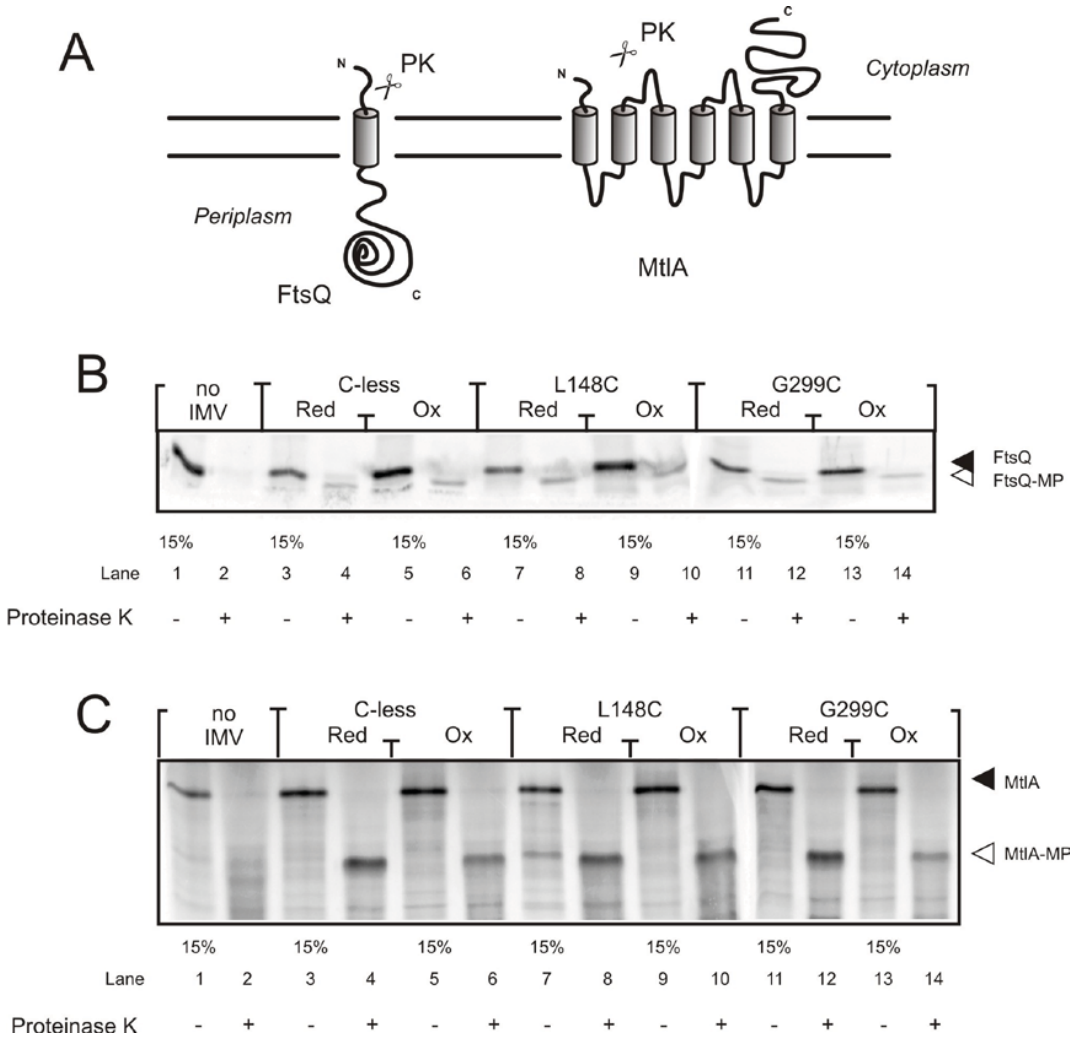

Fig. 6 Membrane insertion of FtsQ and MtlA. (**A**) Schematic representation of the topology of FtsQ and MtlA in the inner membrane. ^{35}S-labeled FtsQ and MtlA were synthesized in a DTT-free *in vitro* transcription-translation system in the presence of IMVs harboring either crosslinked front-to-front SecY dimers or reduced single SecY monomers from the various single cysteine mutants. After 30 minutes at 37 °C proteinase K (0.25 mg/ml) was added to degrade non-inserted material. After inactivation of the protease with TCA precipitation, protease protected material was analyzed by 12% SDS-PAGE and autoradiography. (**B**) Membrane insertion of *in vitro* synthesized FtsQ. (**C**) Membrane insertion of *in vitro* synthesized MtlA. A standard of 15% of the total amount of *in vitro* synthesized membrane protein is indicated.

synthesized *in vitro* as a ^{35}S-methione labeled protein and co-translationally inserted into IMVs that had been treated with either the reductant DTT or the oxidizer NaTT. Under all experimental conditions, limiting amounts of IMVs were used to assure that the level of membrane insertion of FtsQ (and also of MtlA, see below) is dependent on the activity of SecYEG [50,276]. Addition of 5 mM DTT to reduce disulfide-

crosslinked translocons slightly inhibited the *in vitro* synthesis of FtsQ (Compare **Fig. 6**B, *lane 3* to *5*), but did not affect the insertion of FtsQ with IMVs bearing the cysteine-less SecYEG complex (Compare *lane 4* to *6*). Front-to-front crosslinking of the L148C and G299C SecYEG complexes only marginally affected membrane insertion of FtsQ as compared to the cysteine-less SecYEG (**Fig. 6**B, *lanes 10* and *14* vs *lane 6*). This suggests that the front-to-front orientation of the SecYEG dimer is compatible with the co-translational membrane insertion of FtsQ, a process that requires both the ribosome and SecA.

Mannitol permease (MtlA) is a 68 kDa polytopic membrane protein with 6 TMs and no large periplasmic domains (**Fig. 6**A) that does not require SecA or SecB for its insertion [296]. Correct membrane insertion of *in vitro* synthesized ^{35}S-methionine labeled MtlA results in the protease protection of the membrane integrated fragment (30 kDa), and proteolysis of the carboxyl-terminal cytosolic phosphorylation domain [50,296]. Residual amounts of the oxidizer NaTT reduced the efficiency of co-translational membrane insertion of MtlA as evidenced for the cysteine-less SecYEG (**Fig. 6**C, *lane 6* vs *lane 4*). Likewise, membrane insertion of MtlA into NaTT treated IMVs containing L148C and G299C SecYEG complexes was partially inhibited (**Fig. 6**C, *lanes 10*, *14* and *18* compared to *lane 6*). However, the reduction in membrane insertion is very similar to the chemical effect of NaTT on the cysteine-less SecYEG complex. Therefore, these results demonstrate that the co-translational membrane insertion of MtlA proceeds largely unhindered in the disulfide-bond stabilized front-to-front orientation of two translocons.

Discussion

In this study, we have addressed the functionality of the proposed front-to-front orientation of the dimeric translocon in both protein translocation and membrane protein insertion employing a crosslinking approach. On the basis of homology of SecYEG from *E. coli* to SecYEβ from *M. jannaschii* [270] and sequence alignment, we engineered specific cysteine residues in a cysteine-less SecY background. This allowed us to enforce by crosslinking two front-to-front facing translocons with cysteines located on either side of the TM2/TM7 lateral gate region. Importantly, because of the use of mono-cysteines, crosslinking of the front-to-front dimer results in a juxtapositioning of the lateral gates of the opposing translocons in such a manner that they will be unable to fuse to form a so-called consolidated channel (**Fig. 1**B). Remarkably, unlike L148C (TM4), the G299C (TM7) mutant showed a high propensity to spontaneously oxidize into a dimeric arrangement. Also under oxidizing conditions, the G299C mutant yielded the highest levels of crosslinked SecY-SecY dimer.

The crosslinking of two translocons with either L148C (TM2) or G299C (TM7) did not substantially influence the translocation of proOmpA, indicating that both stabilized dimeric states of the translocon are normally active in translocation. This suggests a high plasticity of the front-to-front orientation, as the result was independent of the crosslinking position. In both cases the lateral gates of the channels are unobstructed but they are unable to fuse into a consolidated channel. Since the lateral gate has been implicated in the release of hydrophobic TMs into the lipid membrane, we have also examined the ability of the crosslinked SecY-Y dimers to insert membrane proteins. FtsQ is an integral inner membrane protein that requires

both SecYEG and the SecA motor protein for its insertion [276]. Like proOmpA translocation, insertion of FtsQ was not affected by the front-to-front dimerization (**Fig. 6**B). While the overall synthesis of FtsQ was lower in reduced conditions, the relative insertion levels of FtsQ in crosslinked mutant strains (L148C and G299C) were comparable to that of FtsQ insertion into the cysteine-less mutant where no crosslinking is possible. The same result was obtained for MtlA, a polytopic integral membrane protein that inserts co-translationally in a SecA-independent manner [21,50,138]. Here the overall insertion efficiency of MtlA was affected by oxidative conditions as demonstrated by the effect of NaTT on the insertion of MtlA into cysteine-less IMVs. However, inserting MtlA into IMVs with high levels of front-to-front crosslinked translocons did not lead to a significant reduction in insertion compared to the cysteine-less insertion under oxidative conditions. These data indicate that the dimeric SecYEG complex can function as a binding partner for the ribosome. It should be stressed that the experiments do not address the question if this ribosome interacts with both or only with one of the SecYEG channels in the crosslinked dimer.

Recently, it has been suggested that two translocons co-operate in translocation. One translocon binds the SecA motor protein that would “feed” the preprotein through the pore of the adjacent translocon. According to this model, the functional state of the translocon corresponds to a SecYEG dimer, while translocation occurs through a single pore [201]. However, biochemical and structural analysis of the *T. maritima* SecYEG translocon [78,311] suggest that the SecA motor protein inserts protein substrates into a single SecYEG translocon to which it also binds to. This raises the question if both translocons within a dimeric arrangement would be able to translocate preproteins simultaneously. Previously, it was found that the dimeric state

of SecA is essential for translocation [64,123], while a mutant SecA that exist mostly as a monomer is unable to complement an essential SecA deletion [123]. A later study with engineered SecA dimers also indicated a linear response of dimeric SecA concentration to preprotein translocation levels [122]. With translationally fused SecY dimers it was found that SecA dimers may be required for the initial binding of SecA to the translocon [69]. Furthermore, nucleotides appear to affect the binding stochiometry of SecA to the translocon, as in the presence of ATP, only complexes containing the monomeric SecA could be observed on blue native PAGE. Since the latter only detect very stable complexes, another stoichiometry might be found only transiently. The imposed front-to-front dimeric translocon created in this study effectively translocates preproteins to similar levels as the non-crosslinked translocon (**Fig. 3**A). Importantly, the SecA-dependence (**Fig. 4**A) and ATPase activity of SecA (**Fig. 4**B) remains similar under both reducing and oxidative conditions. While the oxidative environment has an overall negative effect on both protein translocation and the ATPase activity of SecA (at basal levels), no significant reduction in activity has been observed in either case. As previously shown, the stable form of the dimeric SecA obtained by oxidation has an identical activity as native SecA [53]. Our current studies now also shows that this stabilized dimeric form of SecA is also able to functionally associate with the front-to-front translocon (**Fig. 5**B). The exact SecA to SecYEG stoichiometry in such complexes is unknown. Because of space constraints it is however unlikely that both SecA protomers are bound to SecYEG simultaneously.

In conclusion, here we have shown that a stable front-to-front crosslinked translocon is unaffected in its ability to translocate preproteins, and to insert membrane proteins into the membrane. In this arrangement, the position of the

disulfide crosslinked lateral gates excludes the possibility of the formation of a consolidated pore during translocation. The spontaneous formation of a disulfide bond between the TM7 located cysteine residues suggests that this is a favorable site of interaction between opposing lateral gates when in contact. However, since this crosslink is functionally not distinct from a dimeric translocon crosslinked via a cysteine residue in TM2, it appears that the dimeric interface is rather promiscuous. Since the translocon can also be readily crosslinked in a back-to-back orientation, it seems that there is no unique contact interface and that various orientations are possible. Importantly, the current study shows no signs of cooperativity between the translocons within this dimeric arrangement indicating that the proposed dimerization is not a rate-determining step in translocation.

Experimental procedures

Chemicals and biochemicals

Purification of SecA, SecB and proOmpA as well as the isolation of inner membrane vesicles (IMVs) containing overproduced levels of SecYEG were performed as described [271]. ProOmpA (S245C) was labeled with fluorescein maleimide (Invitrogen^{TM}) as described [51]. Sodium tetrathionate (NaTT) was obtained from Sigma-Aldrich while the reducing agent 1,4-dithiothreitol (DTT) was obtained from Roche Applied Science. Enzymes for DNA manipulation were obtained from Promega, Roche and Fermentas and all other chemicals were from Sigma-Aldrich.

Bacterial strains and plasmids

All strains and plasmids used are listed in **Table 1**. All DNA manipulations were performed using *E. coli* DH5α to maintain plasmids and constructs. Cysteine mutations were introduced into a Cys-less SecY using the Stratagene QuikChange® site-directed mutagenesis kit using plasmid pEK1 as template. Mutations were confirmed by sequencing. Plasmids expressing the mutated SecYEG complex were created by exchanging the *Nco*I-*Cla*I *secY* fragment in pEK20 by the cysteine containing *Nco*I-*Cla*I *secY* fragment of the pEK1 derivative. *E. coli* strain SF100 or NN100 (SF100, *unc*$^-$) was used for the overproduction of the various SecYEG complexes.

Chemical crosslinking

IMVs containing overproduced levels of the SecYEG mutants were diluted to 2.5 mg/ml, whereupon the cross-linker NaTT (final concentration 5 mM) or the reducing agent DTT (final concentration 20 mM) was added. For optimal crosslinking, reactions were performed in a volume of 30 µl and when needed multiple reactions were pooled for analysis. After 30 minutes at 37 °C, IMVs were sedimented through a 0.8 M sucrose cushion in 50 mM Tris-HCl, pH 8 (20 minutes 80,000 rpm, TLA 120.1 rotor, 4 ºC). The IMVs were resuspended in 50 mM Tris-HCl pH 8, 20% glycerol (crosslinked samples) or 50 mM Tris-HCl pH 8, 20% glycerol, 5 mM DTT (reduced samples).

Table 1 **Overview of strains and plasmids used in this study**

Strain / Plasmid	***Relevant characteristic***	***Source***
E. coli DH5α	*supE44, ΔlacU169 (Δ80lacZ_M15) hsdR17, recA1, endA1, gyrA96 thi-1, relA1*	[96]
E. coli SF100	*F⁻, ΔlacX74, galE, galK, thi, rpsL, strA, ΔphoA(pvuII), ΔompT*	[16]
E. coli NN100	SF100, *unc⁻*	[195]
E. coli BL21 (DE3)	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE2 (Cam^R)	Novagen
Rosetta		
pBSKftsq	FtsQ	[276]
pET20MtlA	MtlA	[50]
pET36	proOmpA(S245C)	F. Bonardi, unpublished data
pEK1	Cysteine-less SecY	[279]
pEK20	Cysteine-less SecYEG	[279]
pRFY1	SecY(L148C)EG	This study
pRFY2	SecY(G299C)EG	This study
pMKL18	SecA wild-type	[271]

Isolation of oxidized SecA

SecA was overproduced in *E. coli* DH5 using pMKL18 (**Table 1**) as described [271]. Purified SecA was concentrated to a final concentration of 0.7 mg/ml using a Centriprep YM-50 (Millipore, USA). Oxidation of SecA was performed as described [52]. Briefly, prior to crosslinking, 75 µl of SecA was incubated for 30 minutes on ice in the presence of 2 mM EDTA to remove the divalent ions. EDTA was removed using a Micro Biospin P-6 column (BioRad) pre-flushed with buffer (1 mg/ml BSA, 50 mM Tris-HCl, pH 8, and 10% glycerol). Next, the SecA was oxidized by the addition of 1 mM Cu^{2+}(Phenanthroline)$_3$ for 1 hour at room temperature, and excess Cu^{2+}(Phenanthroline)$_3$ was removed using a Micro Biospin P-6 column.

Translocation ATPase assay

The SecA ATPase activity during translocation was determined by measuring the amount of released free phosphate using the malachite green assay [158]. Measurements were done in triplicate and corrected for background ATPase activity.

In vitro transcription-translation-insertion reaction

As disulfide bonds are easily reduced, an *in vitro* transcription-translation system was developed that was devoid of reducing agents. A cell lysate was prepared by inoculating 50 ml double strength (2x) YPTG media [135] with a single colony of BL21 (DE3) Rosetta (Novagen). After overnight incubation at 37 ºC (250 rpm) the pre-culture was used to inoculate a 4 liter Erlenmeyer containing 1 liter of 2x YPTG

media [135]. When the OD_{600} reached a value of 0.6, the culture was chilled in an ice-water bath. Cells were harvested by centrifugation (15 min at 7,500 rpm JLA 10-500 rotor; 4 ºC), washed with buffer A (10 mM Tris-acetate, pH 8.0, 14 mM $Mg(OAc)_2$, 60 mM KCl, and 50 µg/ml PMSF) and finally suspended in 2 ml buffer A per gram of cells. Cells were lysed by passage through a cell disruptor (Constant Cell Disruption Systems [UK]; 10,000 psi). Cell debris was removed by centrifugation (10,000 rpm, SS34 rotor, 4 ºC). The supernatant was transferred to an MLA-80 tube and centrifuged for 30 minutes at 25,000 rpm in a MLA-80 rotor (4 ºC). The supernatant was transferred to a 5 ml falcon tube, supplemented with 55 mM sodium pyruvate, 45 µM coenzyme A, and 110 µM nicotinamide adenine dinucleotide (NAD). After 90 minutes in a water bath (37 ºC) in the dark, membranes were removed by ultracentrifugation (30 minutes 52,000 rpm MLA-80 rotor, 4 °C). The cell lysate was dialyzed for 24 hours against 1 liter of buffer A without PMSF (4 ºC, molecular weight cut-off of 6 to 8 kDa). In this period, the buffer was replaced three times. After dialysis, the lysate was aliquoted, snap-freezed in liquid nitrogen and stored at -80 ºC. DTT-free *in vitro* transcription-translation reactions were performed as described [120] using T7 polymerase from Fermentas and addition of the Easytag express protein labeling mix (Perkin Elmer). For membrane insertion reactions the reaction mixture was supplemented with IMVs at 5 µg/per reaction [271].

Other techniques

In vitro translocation of proOmpA was performed as described [51] with 2 µg of IMVs. Translocated protease resistant proOmpA was separated by SDS-PAGE and visualized with a Roche Lumi-Imager F1 using a cutoff filter of 520 nm. Protein

concentrations were determined with the Bio-Rad RC DC protein assay kit using BSA as a standard.

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Summary and concluding remarks

Introduction

In a bacterium like *Escherichia coli*, about 25 % of all proteins of the proteome must cross at least one lipid barrier to reach their proper location in the cell, while approximately 20% of all proteins are membrane proteins that need to be inserted into a lipid bilayer. The Sec translocase acts as the major facilitator for protein translocation and membrane protein insertion across or into the lipid bilayer. A major question therefore is: how can a single protein complex catalyze such seemingly opposite functions? The translocase needs to discriminate between apolar and polar polypeptide domains and handle these without interfering with the barrier function of the membrane. The Sec translocase is conserved across all domains of life and can be found in the inner membrane of Bacteria and Archaea, the endoplasmic reticulum membrane in Eukaryotes, as well as in the thylakoid membranes of photosynthetic Eukarya [209]. It consists of a heterotrimeric protein complex designated as SecYEG in *E. coli* and can act in concert with various membrane proteins as well as peripherally bound proteins to facilitate the co- or post-translational insertion and translocation of proteins [264]. SecA is a motor protein that facilitates the translocation of preproteins across the SecYEG channel using ATP hydrolysis as an energy source. In this process SecA likely 'pushes' polypeptide segments across the membrane via the SecYEG channel. SecA also plays a role in the translocation of large periplasmic domains of integral membrane proteins [65]. In the eukaryotic translocase, the motor protein BiP associates with the translocon at the

interior of the endoplasmatic membrane and likely "pulls" the protein through the channel [202]. For co-translational membrane protein insertion, the translocon associates with the ribosome, and membrane insertion is coupled to chain elongation at the ribosome. Another conserved and essential protein that can act independently as well as in concert with the translocon is YidC. YidC is a member of the Oxa family of membrane proteins [229] with homologs in mitochondria and chloroplasts [151] Initially, phage proteins were found to utilize YidC for their insertion into the inner membrane of *E. coli* [230], but later small subunits of energy transducing complexes were shown to be the native substrates for YidC [278]. In the late 1990's and early 2000's the focus was on obtaining structural and ultimately functional information on the hetero-trimeric translocon utilizing biochemical approaches. In 2004, the crystal structure of a translocon of the archaeon *Methanocaldococcus jannaschii* was published [270] (**Fig. 2**A). While the archaeal complex, known as SecYEβ, has two subunits closely related to the eubacterial translocon (SecY and SecE), the third subunit, Secβ, is more closely related to its eukaryotic counterpart. The translocon is arranged in a clamshell-like structure where TMs 1-5 and 6-10 of SecY form the encompassing arms of the clamshell when viewed from the top while SecE acts as a brace on the "back" side of the translocon. On the anterior side of the channel a plug domain formed by TM2b closes off the channel and together with a hydrophobic constriction towards the middle of the channel, it most likely functions as a structural block to prevent ion loss when the translocon is not in use [270]. The "front" of the channel has been proposed to be involved in the release of signal peptides of translocated proteins (**Fig. 2**A), as well as a lateral gate for the release of transmembrane segments of membrane inserting proteins [270]. The structural information of the archaeal translocon has stimulated further studies on the structure-

function relationship of the subunits of the translocon, and these now provide a first molecular understanding of the mechanism of channel opening and translocation.

The translocon and YidC act cooperatively in inserting an essential subunit of the cytochrome *bo* quinol oxidase

In a recent study it was shown that components of major energy-transducing complexes in *E. coli* require YidC for their functional assembly [278]. A subsequent *in vitro* study revealed that F_0c, a subunit of the F_1F_0 ATP synthase, requires only YidC for its proper insertion into the inner membrane of *E. coli* [274]. While the insertion of a subset of small inner membrane proteins was shown to be solely dependent on YidC [41,230], its role in membrane proteins that insert via the translocon was poorly understood. YidC can be crosslinked to transmembrane segments of membrane proteins that inserted via the translocon [111-113,240]. However, for such membrane proteins, no direct requirement of YidC for membrane insertion could be demonstrated [229]. In **Chapter 2** we determined the minimal requirements for the membrane insertion of CyoA, also known as the quinol binding subunit of the bo_3 quinol oxidase complex. CyoA was shown to be one of the proteins severely affected by a depletion of YidC *in vivo* [278]. Since CyoA contains a large periplasmic domain that needs to be translocated across the inner membrane, it was suggested that both YidC and SecYEG might be involved in the insertion of this membrane protein. Indeed, by functional reconstitution, we could demonstrate that CyoA insertion into the inner membrane (IM) depends on both YidC and SecYEG. Furthermore, we showed that CyoA requires SecA for proper insertion, likely for the translocation of the periplasmic domain. In contrast to most proteins that are targeted

for translocation, the insertion of CyoA is independent of the proton motive force (PMF). Recently the PMF-independent insertion of CyoA was found to be due to an overall neutral charge of the periplasmic domain [38]. Based on the results described in **Chapter 2** we propose a model for the membrane insertion of CyoA. In the first step of this model, the signal sequence and the first TM domain of CyoA insert into the membrane as a helical hairpin (**Fig. 1**). Next, the cysteine residue at the +1 position of the N-terminus of the mature domain is lipid modified and the signal sequence removed by signal peptidase II. Afterwards, TM2 of CyoA inserts into SecYEG followed by the translocation of the large periplasmic domain with the help of SecA. The specific time-point and role of YidC in the insertion of CyoA is unknown. One could speculate that YidC might be involved in the catalytic removal of the TM segments from the translocon (**Fig. 1**), or alternatively assists in the folding of the loop structure between TM1 and TM2 of the mature CyoA. This step would bear resemblance to the role of YidC in the insertion of F_0c and M13. Interestingly, two other studies investigated the membrane insertion of CyoA using different experimental approaches. Celebi and co-workers [39] found by utilizing truncated N- and C-terminal constructs of CyoA that the N-terminal region, consisting of the signal sequence and first TM, is solely dependent on YidC for its insertion into the membrane while the C-terminal domain, TM2 and the periplasmic domain, requires both YidC and SecYEG. However, results with truncated proteins should be interpreted with caution, as their behavior cannot be directly extrapolated to that of the full-length protein. For example, the N-terminal truncate might be recognized by YidC as an M13 or F_0c-like protein and therefore inserts in the absence of the translocon, whereas in the presence of the translocon, the ribosome might associate with the translocon and insertion may take place at both YidC and the translocon.

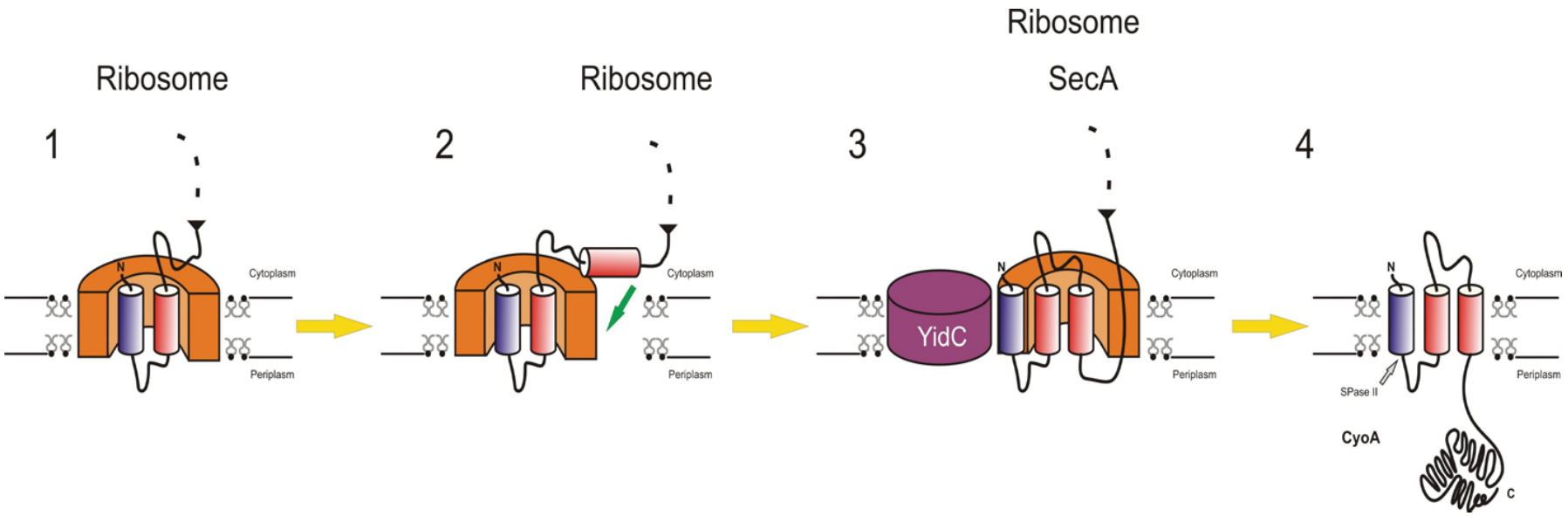

Fig. 1 Schematic representation of the proposed model for the insertion of CyoA via SecYEG and YidC. (**1**) The signal sequence and the first TM domain of the mature CyoA insert co-translationally as a helical hairpin into SecYEG. (**2**) Next, the second TM domain of CyoA is inserted followed by the large periplasmic domain that is translocated via the translocon utilizing SecA as the motor protein and ATP as energy source (**3**). YidC might play a role in catalyzing the release of the TM domains from the Sec-translocon or alternatively ensure the correct topology and folding of the first two TM segments of CyoA (**3**). The cysteine at the +1 position of the N-terminus of the CyoA mature domain is lipid modified after the removal of the first signal-TM domain by signal peptidase II (Spase II) (**4**). The exact time point of interaction of YidC and Spase II is unknown.

Using a similar approach, van Bloois and co-workers [268] suggested that SRP does not bind the signal sequence, but instead interacts with the first TM of the mature CyoA, thereby resulting in the primary insertion of the protein via YidC whereupon the C-terminal domain is inserted by SecYEG. Interestingly, a follow-up study by Celebi and co-workers [38] reported that both the signal peptide as well as the N-terminal membrane anchor domain are important for the translocation of the first periplasmic loop, giving support to the hypothesis that TM1 and TM2 of CyoA insert as a helical hairpin. Furthermore, they also reported that the reason for the lack of a PMF-dependence for CyoA insertion was due to the overall neutral charge of the periplasmic domain as opposed to subunit II of cytochrome c oxidase in mitochondria which is negatively charged and dependent on the PMF [103,106]. By introducing negatively charged amino acids into the hydrophilic periplasmic domain of CyoA they were able to make insertion of this segment PMF dependent [38]. It was also shown that the insertion of CyoA occurs sequentially, wherein the insertion of the C-terminal domain of CyoA is dependent on the correct insertion of the N-terminal domain. These data further show that results with truncated proteins should be taken with caution.

Regardless of the model of insertion, it is clear that there is a direct and specific communication and transfer of proteins between YidC and the translocon. In order to resolve the exact steps and their order, carefully designed experiments are needed. Furthermore, YidC has been suggested to be involved in the assembly of larger respiratory chain complexes and it would be of great importance to elucidate these enigmatic processes.

The lateral gate of SecYEG opens to facilitate protein translocation but not membrane protein insertion

In **Chapter 3**, we investigated the lateral release of TM segments of membrane proteins that are inserted via the translocon. Investigating this step would not only help to elucidate where and how TM segments exit the translocon, but it would also provide a further understanding of the functional significance of an interaction between YidC and SecYEG.

Using the crystal structure (**Fig. 2**A) of the *M. jannaschii* SecYEβ [270] and a homology model of the *E. coli* SecYEG, we introduced cysteine pairs in the proposed lateral gate of the translocon of *E. coli*. This enabled crosslinking of TM2b to TM7 and an investigation of the role of lateral gate opening during protein translocation and membrane protein insertion. Importantly, utilizing cysteine-specific crosslinkers of differing lengths we were able to show that the opening of the lateral gate region is essential for the translocation of preproteins across the translocon. In agreement with our data, the SecA-bound crystal structure (**Fig. 2**B) of the translocon from *T. maritima* [311], displayed a semi-open state of the lateral gate and an indication that a two-helix finger of SecA inserts into the translocon during protein translocation [78]. In addition, Tsukazaki *et al* [259] presented a crystal structure of an antibody-stabilized pre-open state of SecYE from *T. thermophilus* which also suggested the opening of the lateral gate area upon binding of SecA. Interestingly, whereas the formation of a disulfide bond (2 Å) between two cysteines in the lateral gate abolished protein translocation, introducing bifunctional crosslinkers of 5 Å or longer did not affect protein translocation. These data suggest that translocation occurs via a single translocon as the presence of a crosslinkers in the mid-section of the lateral gate area

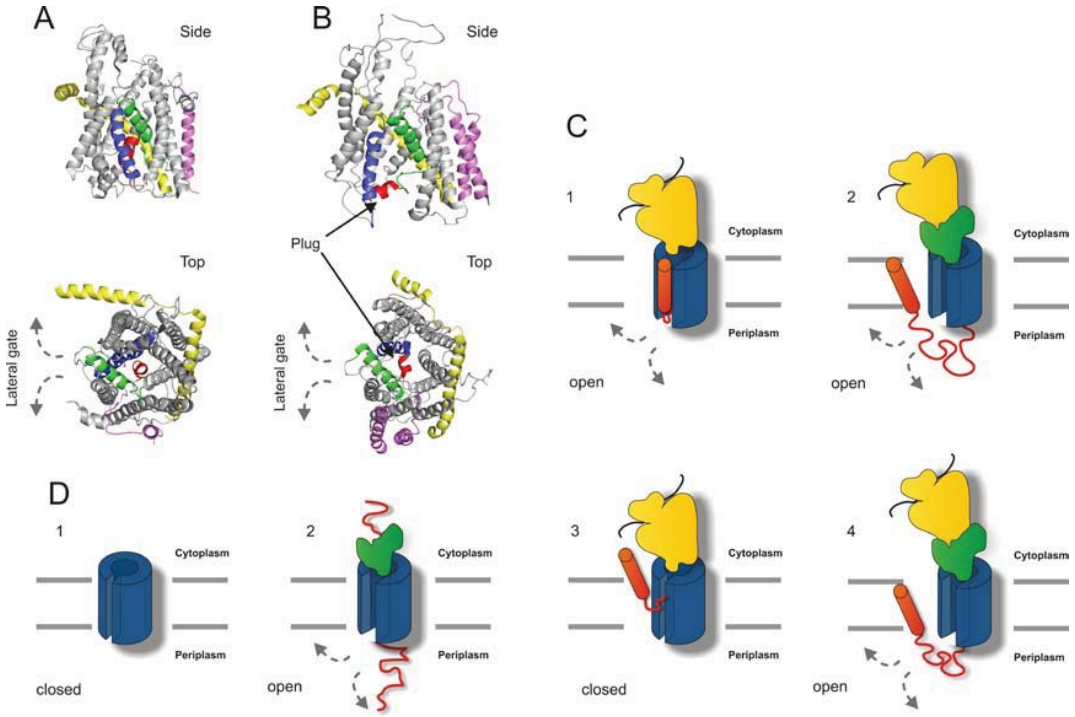

Fig. 2 Structure of the Sec translocase and models for the mechanism of channel opening by a signal sequence or TM domains. (A) Side view and top view (cytosolic face) of the crystal structure of SecYEβ from *M. jannaschii* (PDB coordinates: 1RHZ) [270]. (B) Side view and top view (cytosolic face) of the crystal structure of SecA bound SecYEG from *Thermotoga maritima* (PDB: 3DIN) [311]. Indicated in both structures are the plug (*red*), SecE (*yellow*), SecG/β (*magenta*) as well as TM2 (*green*) and TM7 (*blue*) forming the proposed lateral gate. (C) Model for the membrane insertion of TM segments requiring the opening of the lateral gate [270] (C1). For proteins containing periplasmic domains, translocation would continue through the centre of the translocon after the lateral release of the TM domain (or signal sequence) (C2). (D) Model for the membrane insertion of TM segments without opening of the lateral gate. D1 represents the closed state of the translocon as depicted in (A). D2 represents the semi-open SecA bound state as depicted in (B) where translocation requires the opening of the lateral gate to accommodate the insertion of SecA. D3 represents the insertion of membrane proteins lacking periplasmic domains, such as MtlA, where lateral gate opening is not required and the TM segments can slide through the top of the TM2/TM7 gate perpendicular to the membrane. Here the plug domain remains in position as depicted in (A). No atomic structural information exists for this state where the ribosome is bound instead of SecA. D4 represents the insertion of membrane proteins containing periplasmic domains that are dependent on SecA for their assembly. Here, the TM segments would insert as shown in D3, while the translocation of the periplasmic domain through the center of the SecYEG channel involves SecA that is required for the central pore opening, a process that is accompanied with the opening of the lateral gate.

precludes the efficient formation of a consolidated pore between two front-to-front orientated translocons. Using a trapped translocation intermediate, we were able to crosslink the lateral gate cysteines by disulfide bond formation or by using bBBr (crosslink distance 5 Å). Crosslinkers of longer length were unable to form a crosslink in the lateral gate when the translocon contained a translocation intermediate

suggesting that the lateral gate is closed during ongoing translocation of a preprotein. Moreover, the ATPase activity of SecA was not activated upon the addition of a preprotein when the lateral gate was closed via a disulfide bond. Taken together, the data presented in **Chapter 3** suggest that lateral gate opening and the activation of the SecA ATPase activity are coordinated events suggesting an allosteric mechanism for the channel opening (**Fig. 2**B).

In the **Appendix to Chapter 3**, we analyzed the insertion of membrane proteins under conditions that the two cysteines in the lateral gate were crosslinked. In this study, we utilized three membrane proteins, FtsQ, CyoA and MtlA as substrates that differ in terms of their insertion requirements. FtsQ requires the translocon and SecA for its proper insertion as it contains a large periplasmic domain. CyoA also requires the translocon and SecA for its insertion, but in addition as shown in **Chapter 2** also requires YidC for its correct insertion into the IM (**Fig. 1** and **Fig. 2**D). Finally, MtlA is a polytopic membrane protein that requires the translocon for its insertion, but does not require SecA as it lacks large periplasmic domains (**Fig. 2**D). As shown for the translocation of proOmpA, we found that the insertion of FtsQ and CyoA is inhibited by crosslinkers of 5 Å or shorter. We speculate that the opening of the lateral gate is primarily needed for the translocation of the periplasmic domain. However, with the co-translational insertion of MtlA, which does not require SecA, neither chemical crosslinkers nor a disulfide bond could inhibit the insertion of MtlA into IMVs. This suggests that TM segments of membrane proteins can leave the translocon without the need for the opening of the lateral gate between TM2b and TM7 (**Fig. 2**D). One explanation could be that the TM segments never enter the translocon past the hydrophobic constriction ring and leave the translocon almost perpendicular to the lipid bilayer over the slanted TM2 and TM7 segments (**Fig. 2**D).

Furthermore, our data showed that crosslinking the lateral gate with the semi-flexible crosslinker BMH negatively affects the insertion of polytopic membrane proteins while it does not affect protein translocation. We speculate that this longer crosslinker prevents the lateral gate to close completely and this might be incompatible with the insertion of the TM segments of membrane proteins. Based on the results presented in **Chapter 3** and its accompanying appendix we propose that the TM segments enter the translocon up to the hydrophobic constriction without displacing the plug domain. From here the TM segments slide into the lipid bilayer at the cytosolic face of the translocon (**Fig. 2**D). In contrast, signal sequences and periplasmic domains are hypothesized to make use of the complete lateral gate where the flexibility in this area is essential for the functioning and ATP hydrolysis of SecA (**Fig. 2**C and **Fig. 2**D). While proteins containing only TM segments can clearly be inserted without the need for an open lateral gate, this gate needs to open for the insertion of proteins containing periplasmic domains such as CyoA and FtsQ and thus require SecA for translocation. If the TM segment slide over the interfering crosslinker and the rest of the protein is translocated through the channel, one might envisage a situation where the protein gets stuck in the channel with a TM segment outside the translocon and a periplasmic domain remaining on the inside. As the membrane protein insertion efficiency is similar to that of the wild-type vesicles it might be that the translocon/ribosome/SecA complex deals with such proteins differently (**Fig. 2**D). In fact, it is still unclear how the ribosome hands over the synthesized protein to SecA once a large enough polar domain is encountered. Furthermore, what would happen if a protein contains a C-terminal TM domain following a large periplasmic domain? How does SecA deal with these hydrophobic segments? What is the role of the ribosome in such processes, or does it occur entirely post-translational? Clearly many steps in membrane protein

biogenesis are unclear also considering the wide range of potential types of substrate that need to enter the membrane via the translocase. Therefore, further studies into the mechanism by which TM segments leave the lateral gate, as well as the steps where the ribosome, the translocon, SecA and other ancillary proteins such as YidC and SecDF(yajC) play a role will be of great interest. For instance, the undisturbed TM insertion of a protein such as MtlA by a translocon with a closed lateral gate raises questions on the potential mechanism by which TM segments are handed over from SecYEG to YidC.

Front-to-front crosslinking of two translocons has no effect on protein translocation and membrane protein insertion

Following the results obtained in **Chapter 3** we were intrigued by the role of the lateral gate in the hypothesized front-to-front oriented SecYEG translocons. Various studies have suggested the existence of either “front-to-front” or “back-to-back” translocons. To elucidate the role of a “front-to-front” arrangement of translocons in translocation and membrane insertion, we introduced cysteines on either side of TM2 and TM7 of the proposed lateral gate to enforce a stable “front-to-front” arrangement of SecYEG translocons. The introduction of a single cysteine residue in SecY is sufficient to juxtapose the lateral gates of the opposing translocons and upon crosslinking would enable the determination of how and where TM segments or signal sequences exit the translocon. In addition, such crosslinked arrangement would also preclude the formation of a consolidated pore. The two single cysteine residues introduced in SecY crosslinked two TM4 segments or two TM7 segments, respectively. With both introduced cysteine residues we found a very

efficient dimer formation which unaffected protein translocation. This suggested that both translocons in a stable front to front orientation are active in translocation. Recently, it was suggested that translocation occurs through a back-to-back arrangement of SecYEG where only one copy of the dimer is active in translocation while the other copy acts as a binding site for SecA [201]. However, we found that crosslinked front-to-front dimeric translocons translocated proteins to similar levels as wild-type or non-crosslinked SecYEG while maintaining the same levels of SecA dependence. In addition, translocation reactions utilizing a dimeric crosslinked SecA together with the front-to-front crosslinked translocon showed no loss of translocation activity. Although we do not know if both SecA protomers in the dimer are simultaneously active or act sequentially, above data fits well with previous studies that found that dimeric SecA is essential for translocation [64,122,123]. We have also investigated the role of the front-to-front dimer in the insertion of membrane proteins. Herein, we used the membrane proteins FtsQ and MtlA as model substrates. Surprisingly, for both the SecA-dependent FtsQ and the SecA-independent MtlA the front-to-front crosslinking did not affect insertion. This also suggests that the dimeric translocon can act as a binding site for the ribosome. It should however be noted that it is difficult to envisage how the ribosome aligns with the channel of either translocon, and therefore it is more likely that the ribosome interacts with only one of the translocons and the other one is not used. In conclusion, our data suggest that the formation of a consolidated pore between two front-to-front oriented translocons is not essential for protein translocation or membrane protein insertion. Moreover, a specific role for the front-to-front or back-to-back SecYEG translocon in catalysis seems elusive for now.

Future Outlook

While many of the underlying mechanistic principles of protein translocation and membrane protein insertion have been elucidated during the last 20 years, many questions remain. Burning topics such as how transmembrane segments exit the translocon, the re-organization of TM segments inside the translocon and the mechanism of transfer of TM segments from SecYEG to YidC during membrane protein biogenesis remain unclear. Furthermore, despite all the experimental results pointing to the different multimeric states of the translocon and the motor protein SecA, the mechanistic role(s) of these states remain elusive. Also the role of other ancillary proteins and complexes such as the SecDF(yajC) complex in both protein translocation and membrane protein insertion is far from clear. In this respect, proteins with attenuating functions in protein translocation/membrane protein insertion will be the most difficult to characterize experimentally. With the availability of several crystal structures of the translocon, directed approaches, such as the ones utilized in this thesis, can now be implemented to unravel the underlying mechanistic principles of protein translocation and membrane protein insertion. Also of great importance are the current opportunities to investigate molecular mechanisms at the single molecule level, thereby giving detailed insight into the physical and chemical reactions involved in each step of the biogenesis pathway. This might lead to the understanding how multimeric protein complexes assemble and specifically what the role, if any, YidC and other proteins play in the assembly of these complexes. While there is ample structural data on SecYEG, so far the other important interacting partner involved in membrane protein biogenesis, YidC, has escaped crystallization. Clearly, while entering an exciting time in the investigation of

the structure-function relationship of the translocase, the techniques developed can also be utilized to study other membranous transport systems for which our mechanistic understanding is lagging behind.

Nederlandse samenvatting

Inleiding

In bacteriën, zoals *Escherichia coli,* wordt ongeveer een kwart van alle eiwitten binnen de cel gesynthetiseerde eiwitten over een of meerdere membranen getransporteerd voordat ze hun uiteindelijke locatie in de cel bereiken. Daarnaast bestaat circa een vijfde van alle eiwitten uit membraaneiwitten, die in het membraan geïnsereerd moeten worden. Beide processen, eiwittranslocatie en membraaneiwitinsertie worden gedreven door een enzym complex dat het Sec translocase wordt genoemd. Een centrale vraag in het onderzoek is hoe dit enkele eiwit complex deze twee schijnbaar tegengestelde functies kan katalyseren. Het translocase moet onderscheid maken tussen water-afstotende en water-aantrekkende delen van het eiwit en deze over de membraan transporteren (transloceren) of in de membraan inserteren zonder te interfereren met de barrière functie van het membraan.

Het Sec translocase is geconserveerd in alle domeinen van het leven. Het komt voor in de binnenmembraan van bacteriën en Archaea, het endoplasmatisch reticulum membraan in eukaryoten en in de thylakoid membraan van fotosynthetische eukaryoten [209]. Het translocon bestaat uit een heterotrimeer membraaneiwit complex dat samenwerkt met verschillende membraaneiwitten en cytosolische eiwitten [264]. In *E. coli* bestaat het translocase uit de eiwitten SecY, SecE en SecG die tesamen het eiwitdoorlaatbare SecYEG complex vormen. Het transport van eiwitten door het SecYEG kanaal wordt gedreven door het motoreiwit SecA. SecA

gebruikt de de energie van ATP binding en hydrolyse om polypeptide ketens door het SecYEG kanaal te 'duwen'. SecA is ook betrokken bij de translocatie van grote periplasmatische domeinen van membraaneiwitten [65]. In tegenstelling tot *E. coli* en andere bacteriën maakt het eukaryote translocon gebruik van het motoreiwit BiP, dat zich aan de binnenkant van de endoplasmatisch reticulum membraan bevindt en dat waarschijnlijk ten koste van ATP binding en hydrolyse eiwitten door het kanaal `trekt` [202]. Wanneer het eiwit in de membraan geïnsereerd moet worden associeert het SecYEG complex met een ribosoom, waaraan het nieuw gevormde membraaneiwit gesynthetiseerd wordt. In dit proces is de groei van de polypeptide keten direct gekoppeld aan de insertie van het nieuw gevormde membraaneiwit in het membraan via het translocon. Tijdens dit proces kan het translocon samenwerken met een ander essentieel,en geconserveerde membraaneiwit, namelijk YidC. Dit eiwit behoort tot de Oxa familie van membraaneiwitten [229] die ondermeer homologen omvat die functioneren in de binnenmembraan van de mitochondriën en de thylakoiden membraan van chloroplasten [151]. YidC kan ook onafhankelijk van het translocon membraaneiwitten inserteren. Als substraten werden in eerste instantie alleen faag eiwitten gevonden die YidC gebruikten om in de *E. coli* binnenmembraan te inserteren [230]. Later bleek vin *E. coli* ook een aantal kleine membraansubeenheden van energie transducerende complexen te fungeren als substraten voor YidC [278].

Rond de eeuwwisseling werd vooral met behulp van biochemische technieken geprobeerd om structurele en functionele informatie over de translocase te krijgen. In 2004 werd de kristalstructuur van het Sec translocase van het archaeon *Methanocaldococcus jannaschii* gepubliceerd [270] **(Fig. 2** A). Dit complex, bekend als SecYEβ, bevat twee subeenheden die nauw verwant zijn aan het

eubacteriële translocase (SecY en SecE). De derde subeenheid, SecG, is nauwer verwant aan het eukaryote translocase. Uit de structuur blijkt dat het translocase een zandlopervormig kanaal in het membraan vormt. De vernauwing in het midden van het kanaal wordt gevormd door hydrofobe aminozuren. In het deel van zandloper dat zich aan de buitenzijde van de cel opent bevindt zich een zogenaamd 'plug-domein' dat wordt gevormd door een kleine alfa helix die terugvouwt in trechtervormige opening. Het plug-domein en de hydrofobe vernauwing functioneren waarschijnlijk als een thermodynamische barrière die voorkomt dat gehydrateerde ionen weg kunnen lekken wanneer het translocase niet in gebruik is [270].

Vanuit de binnenkant van de cel ziet het translocase eruit als een schelp-achtige structuur. De eerste en laatste vijf TM segmenten van SecY vormen de kleppen van de schelp die aan de achterzijde bijeen gehouden worden door SecE. Op basis van de structuur wordt gesuggereerd dat de voorzijde van het kanaal kan openen wanneer een signaal peptide inserteert. Het signaal peptide kan dan het kanaal verlaten via deze zogenaamde laterale uitgang **(Fig. 2** A). De lateral uitgang kan mogelijk ook dienen als een uitgang voor de TM segmenten van membraaneiwitten die op die wijze het hydrofobe deel van het membraan kunnen bereiken [270]. Deze structurele informatie van het translocon van een archaeon heeft het onderzoek aan de structuur-functie relatie van de subeenheden van het translocase gestimuleerd en deze studies hebben tot een vergaand begrip geleidt in het moleculaire werkingsmechanisme van het kanaal tijdens translocatie.

SecYEG en YidC werken samen tijdens de membraaninsertie van een essentiële subeenheid van het cytochroom *bo* quinol oxidase

Onlangs werd aangetoond dat onderdelen van belangrijke energie-transducerende complexen afhankelijk zijn van het YidC eiwit voor hun functionele assemblage [278]. Vervolgens toonde *in vitro* onderzoek aan dat de c-subeenheid van de F_0 sector ,van het F_1F_0 ATP synthase, alleen afhankelijk is van YidC voor de insertie in de binnenmembraan van *E. coli* [274]. Hoewel YidC in staat lijkt zelfstandig een aantal kleine membraaneiwitten in de binnenmembraan te inserteren [41,230], is de exacte rol van YidC in membraaneiwit insertie via SecYEG nog onduidelijk. YidC kan chemisch gekoppeld worden met nieuw gevormde TM segmenten die via SecYEG in het membraan inserteren [111-113,240]. Echter een directe rol van YidC in de Sec-afhankelijke insertie van deze membraaneiwitten is nog niet aangetoond [229]. In **Hoofdstuk 2** hebben we bepaald wat de minimale vereisten zijn voor de membraaninsertie van CyoA, de quinol bindende subeenheid van het bo_3 quinol oxidase complex. CyoA is een membraaneiwit met twee transmembraan segmenten en een groot carboxyl-terminaal periplasmatisch domein. Het wordt gesynthetiseerd met een amino-terminale signaalsequentie die na insertie wordt verwijderd door signaal peptidase II. Eerder onderzoek heeft aangetoond dat CyoA een van de eiwitten waarvan het niveau in de binnenmembraan sterk beïnvloed wordd door de depletie van YidC *in vivo* [278]. Omdat het periplasmatische domein van CyoA over het binnenmembraan getransporteerd moet worden werd gesuggereerd dat YidC en SecYEG beide betrokken zijn bij de insertie. Met behulp van functionele reconstitutie experimenten konden we inderdaad aantonen dat zowel YidC als SecYEG nodig zijn voor de insertie van CyoA. Verder toonden we aan dat de

membraaninsertie van CyoA afhankelijk is van SecA, dat waarschijnlijk nodig is voor de translocatie van het periplasmatische domein. In tegenstelling tot de meeste eiwitten die getransloceerd worden, is de insertie van CyoA niet afhankelijk van de protonen gedreven kracht (PMF). Een recente studie schreef de PMF-onafhankelijke insertie van CyoA toe aan de neutrale lading van het periplasmatische domein [38]. In **Hoofdstuk 2** presenteren we een model voor CyoA membraaninsertie. In de eerste stap van dit model worden de signaalsequentie en de eerste TM domein van CyoA als een haarspeld in het membraan geïnsereerd **(Fig. 1).** Het cysteïne residu direct achter de signaalsequentie wordt gekoppeld aan een lipide waarna de signaalsequentie wordt verwijderd door signaal peptidase II. Vervolgens wordt het tweede TM segment via SecYEG geïnsereerd en wordt het periplasmatische domein van CyoA met behulp van SecA over het membraan getransporteerd. We weten nog niet hoe en wanneer YidC het translocase bij dit proces helpt. Mogelijk is YidC betrokken bij de verwijdering van de TM segmenten uit het translocase **(Fig. 1),** of speelt het een rol bij de vouwing van de structuur tussen de eerste twee TM segmenten van CyoA. Dit zou lijken op de rol die YidC speelt tijdens de insertie van F_0C en M13. De membraaninsertie van CyoA is ook met behulp van andere experimentele benaderingen onderzocht. Celebi en medewerkers [39] hebben met behulp van ingekorte amino- en carboxyl-terminale delen van CyoA gevonden dat het amino-terminale deel, dat uit de signaalsequentie en het eerste TM segment bestaat, uitsluitend afhankelijk is van YidC voor membraaninsertie. Het carboxyl-terminale domein, dat bestaat uit het tweede TM segment en het periplasmatische domein, heeft zowel YidC en SecYEG nodig. Echter resultaten met kunstmatig ingekorte eiwitten moeten voorzichtig geïnterpreteerd worden, omdat hun gedrag niet altijd rechtstreeks geëxtrapoleerd kan worden naar het volledige eiwit. Het is bijvoorbeeld mogelijk dat

het amino-terminale deel van het eiwit door YidC herkend wordt als een soort M13 procoat of F_0c-achtig eiwit en daarom onafhankelijk van de translocase insereert, terwijl in aanwezigheid van de translocase het ribosoom met het translocase zou kunnen associëren waarna insertie kan plaatsvinden via de translocase en YidC.

Met behulp van een soortgelijke aanpak hebben van Bloois en medewerkers [268] voorgesteld dat signaal herkenningseiwit (SRP) niet aan de signaalsequentie maar direct aan het eerste TM segment van CyoA bindt. Dit zou leiden tot insertie van het amino-terminale domein van CyoA via YidC waarna het carboxyl-terminaal domein via SecYEG wordt geïnsereerd. In een vervolgstudie hebben Celebi en medewerkers [38] aangetoond dat zowel het signaal peptide als het amino-terminale membraananker domein van belang zijn voor de translocatie van de eerste periplasmatische lus. Dit onderbouwt de hypothese dat TM1 en TM2 van CyoA als een haarspeld geïnsereerd worden. Daarnaast werd aangetoond dat de PMF-onafhankelijke insertie van CyoA veroorzaakt wordt door de neutrale lading van het periplasmatische domein. In tegenstelling tot *E. coli* CyoA heeft subeenheid II van cytochroom c oxidase in mitochondria een negatief geladen domain en is de membraaninsertie van dit eiwit afhankelijk van de PMF [103,106]. Door negatief geladen aminozuren in het periplasmatische domein van CyoA te brengen kon de insertie van CyoA PMF afhankelijk gemaakt worden [38]. Tenslotte werd aangetoond dat de insertie van het carboxyl-terminale domein van CyoA afhankelijk is van de correcte insertie van het amino-terminale domein. Dit laatste bewijst nogmaals dat resultaten met ingekorte eiwitten met de nodige voorzichtigheid geïnterpreteerd moeten worden.

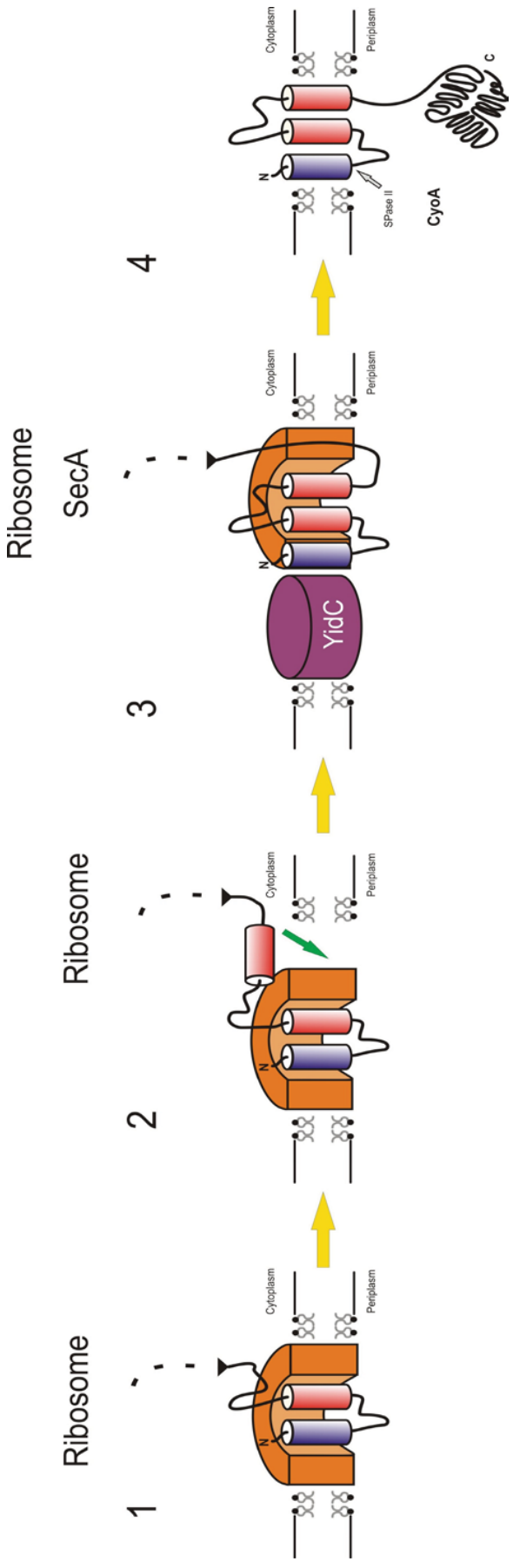

Fig. 1 Schematische weergave van het voorgestelde model voor de insertie van CyoA via SecYEG en YidC. **(1)** De signaalsequentie en het eerste TM segment van CyoA inserteren co-translationeel, als een haarspeld, in SecYEG. **(2)** Vervolgens, wordt het tweede TM segment geïnsereerd, waarna het periplasmatische domein door het translocon over de membraan wordt getransporteerd. Het translocon wordt hierbij geholpen door SecA als motor eiwit en ATP als energiebron **(3).** YidC zou een rol kunnen spelen bij het vrijkomen van de TM domeinen uit de Sec-translocase of zorgen voor de juiste topologie en vouwing van de eerste twee TM segmenten van CyoA **(3).** Na verwijdering van de signaalsequentie door signaal peptidase II (SPase II) wordt een lipide gebonden aan de cysteïne die zich direct achter de signaalsequentie bevindt **(4).** Het exacte tijdstip van de interactie van YidC en SPase II is onbekend.

Ongeacht het model, is het duidelijk dat er een directe en specifieke communicatie is met betrekking tot de overdracht van eiwitten tussen YidC en SecYEG. Om de enzymologie van die stappen nauwkeurig op te lossen is vervolgonderzoek nodig. Immers YidC blijkt betrokken te zijn bij de assemblage van grote ademhalingsketen complexen en daarom is het van groot belang het raadselachtige proces van membraaninsertie verder op te helderen.

De laterale uitgang van SecYEG is betrokken bij eiwittranslocatie maar niet bij membraaneiwit insertie

In **Hoofdstuk 3** hebben we de rol van de hypothetische laterale uitgang van SecYEG bij het vrijkomen van TM segmenten uit het kanaal onderzocht. Inzicht in deze stap helpt niet alleen te begrijpen waar en hoe TM segmenten het translocase verlaten, maar leidt ook tot een beter begrip van de functionele interactie tussen YidC en SecYEG.

Aan de hand van de kristalstructuur **(Fig. 2** A) van het *M. jannaschii* SecYEβ complex [270] en een homologie model van *E. coli* SecYEG hebben we cysteïnes geïntroduceerd aan weerszijden van de laterale uitgang van het translocon. Dit stelde ons in staat om TM segment 7 aan TM segment 2b te koppelen (crosslinken) om de rol van de laterale uitgang tijdens eiwittranslocatie en membraaneiwitinsertie te onderzoeken. Door gebruik te maken van cysteïne-specifieke chemische crosslinkers met verschillende lengtes konden we aantonen dat de opening van de laterale uitgang essentieel is voor het transport van eiwitten door het translocatiekanaal. Inderdaad, in de SecA-gebonden kristalstructuur **(Fig. 2** B) van de translocase van *T. maritima* [311] is de laterale uitgang in een semi-geopende toestand. Deze

structuur suggereerde bovendien dat de twee-helix vinger van SecA die verondersteld wordt een belangrijke rol te spelen tijdens eiwit-translocatie, in het translocase insereert [78]. Daarnaast hebben Tsukazaki *et al.* [259] een kristalstructuur gepresenteerd van een door een antilichaam gestabiliseerde pre-open toestand van SecYE van *T. thermophilus*. Deze structuur suggereert ook dat de laterale uitgang opent wanneer SecA aan SecYEG bindt. Interessant genoeg bleek dat een disulfide brug (2 Å) tussen de twee cysteïnes in de laterale uitgang, eiwittranslocatie verhindert, terwijl chemische crosslinkers met een verbindingen van 5 Å of langer relatief weinig invloed hebben op eiwit translocatie.De crosslinkers in het midden van de laterale uitgang verhindert dat twee translocons die via de voorkant met elkaar geassocieerd raken en daarmee een gezamenlijk kanaal kunnen vormen. Dit suggereert dat translocatie plaats vindt via een enkel translocon.

Wanneer het translocase een deels getransloceerd substraateiwit (een translocatie intermediair) bevatte konden de cysteïnes aan weerszijden van de laterale uitgang nog steeds een disulfide bruggen vormen of worden gekoppeld met de crosslinker bBBr (5 Å). Echter onder die condities konden de cysteïnes niet gekoppeld worden met langere crosslinkers, wat suggereert dat de laterale uitgang gesloten is tijdens translocatie. Wanneer de laterale uitgang door middel van een disulfide brug gesloten werd kon de ATPase activiteit van SecA niet meer geactiveerd worden door precursoreiwitten. De resultaten die beschreven staan in **Hoofdstuk 3** suggereren dat de opening van de laterale uitgang en de SecA ATPase activiteit gecoördineerde gebeurtenissen zijn die duiden op een allosterisch mechanisme voor de opening van het SecYEG kanaal **(Fig. 2** B).

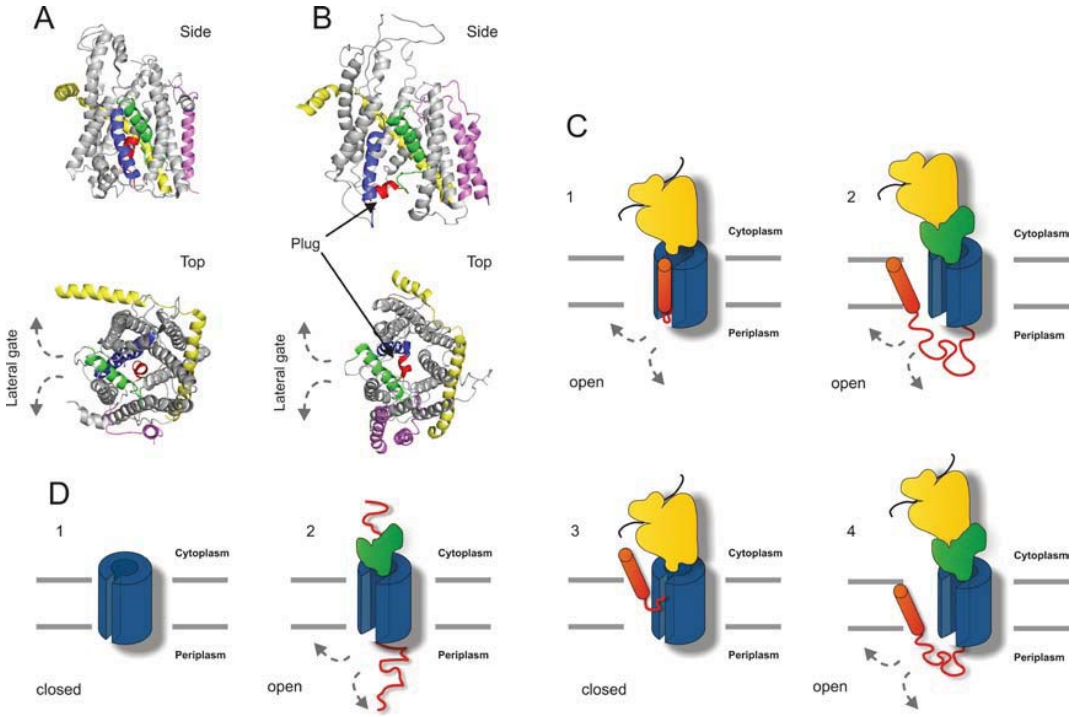

Fig. 2 Structuur van het Sec translocon, en modellen voor het mechanisme van de opening van het kanaal door signaalsequentie of TM domeinen. (A) Zijaanzicht en bovenaanzicht (vanuit het cytosol) van de kristalstructuur van SecYEβ van *M. jannaschii* (PDB coördinaten: 1RHZ) [270]. (B) Zijaanzicht en bovenaanzicht van de kristalstructuur van het SecA gebonden SecYEG van *Thermotoga maritima* (PDB: 3DIN) [311]. In beide structuren zijn de plug *(rood),* SecE *(geel)* en SecG / β *(magenta)* aangegeven, alsmede TM2 *(groen)* en TM7 *(blauw)* die de voorgestelde laterale uitgang vormen. (C) Model voor membraan insertie van TM segmenten zonder de opening van de laterale uitgang [270] (C1). Voor membraaneiwitten met periplasmatische domeinen, zou, na het lateraal vrijkomen van het TM domein (of de signaalsequentie), de translocatie verder verlopen door het centrum van de translocase (C2). (D) Model voor membraaninsertie van TM segmenten zonder opening van de laterale uitgang. D1 staat voor de gesloten toestand van de translocase zoals weergegeven in (A). D2 staat voor de semi-open SecA gebonden toestand zoals weergegeven in (B) waar translocatie de opening van de laterale uitgang vereist door de invoeging van SecA. D3 verbeeldt de insertie van membraaneiwitten zonder periplasmatische domeinen, zoals MtlA, waar laterale uitgang van de uitgang niet nodig is en de TM-segmenten, via de top van de TM2/TM7 uitgang, loodrecht op de membraan kunnen schuiven. Zoals afgebeeld in (A) blijft het plug domein hier op dezelfde plaats (A). Er bestaat geen structurele informatie voor deze toestand, waarbij het ribosoom is gebonden in plaats van SecA. D4 verbeeldt de insertie van membraaneiwitten die periplasmatische domeinen bevatten en daardoor afhankelijk zijn van SecA voor hun assemblage. Hier zouden TM segmenten mogelijk inserteren zoals afgebeeld in D3, terwijl de translocatie van het periplasmatische domein door het centrum van de SecYEG kanaal plaatsvindt. SecA is nodig voor de opening van de centrale porie, een proces dat gepaard gaat met de opening van de laterale uitgang.

In de **Appendix van hoofdstuk 3** analyseerden we het effect van koppeling van cysteïnes in de laterale uitgang op de insertie van membraaneiwitten. Hiervoor gebruikten we drie membraaneiwitten (MtlA, FtsQ en CyoA) die verschillen in hun vereisten voor membraaninsertie. FtsQ heeft naast het translocon SecA nodig voor de translocatie van het grote periplasmatisch domein over het membraan. Insertie van

CyoA vereist ook SecYEG en SecA, maar daarnaast, zoals weergegeven in **Hoofdstuk 2**, is ook YidC nodig voor correcte insertie **(Fig. 1** en **Fig. 2** D). MtlA is een polytoop membraaneiwit zonder grote periplasmatische domeinen dat alleen het translocase nodig heeft voor insertie **(Fig. 2**D). Net als bij de translocatie van proOmpA (**Hoofdstuk 3**), vonden we dat de insertie van FtsQ en CyoA geremd wordt door crosslinkers van 5 Å of korter en we speculeren daarom dat de opening van de laterale uitgang in de eerste plaats nodig is voor de translocatie van het periplasmatische domein. Verrassend genoeg bleek de insertie van MtlA niet geremd te worden door de introductie van een disulfide brug of chemische koppeling van de laterale uitgang. Dit suggereert dat TM segmenten van membraaneiwitten het translocon kunnen verlaten zonder de opening van de laterale uitgang tussen TM2b en TM7 plaatsvindt **(Fig. 2** D). Een verklaring zou kunnen zijn dat TM-segmenten nooit dieper in het SecYEG kanaal komen dan tot de hydrofobe vernauwing en dat ze het translocase bijna loodrecht ten opzichte van de lipide bilaag over de schuine TM2 en TM7 segmenten verlaten **(Fig. 2** D). Verder blijkt uit onze resultaten dat de chemische koppeling van de laterale uitgang met de semi-flexibele crosslinker BMH een negatieve invloed heeft op de insertie van polytope membraaneiwitten, terwijl eiwittranslocatie niet gehinderd wordt. We speculeren dat deze langere crosslinker de volledige sluiting van de laterale uitgang verhindert, wat onverenigbaar is met de membraaninserteren van de hydrophobe TM segmenten. Gebaseerd op de resultaten gepresenteerd in **Hoofdstuk 3** en de bijbehorende appendix stellen wij voor dat TM-segmenten het translocon in gaan tot bij de hydrofobe vernauwing en dat het plug-domein hierbij op zijn plaats blijft. Vervolgens schuiven de TM segmenten in de lipide bilaag aan de cytosolische kant van het translocase **(Fig. 2**D). In tegenstelling tot TM segmenten, maken signaalsequenties en periplasmatische domeinen gebruik

van de volledige laterale uitgang waarbij de flexibiliteit in dit deel van het eiwittranslocatie kanaal essentieel is voor de functie en ATPase activiteit van SecA **(Fig. 2** C en **Fig. 2** D). In tegenstelling, eiwitten met alleen TM segmenten kunnen geïnsereerdworden zonder opening van de laterale uitgang maar indien er grote periplasmatische domeinen getransloceerd moeten worden zal SecA nodig zijn waardoor er alsnog een opening van de laterale uitgang plaatsvindt. Wanneer een TM-segment over het gecrosslinkte deel van de translocase glijdt en de rest van het eiwit door het kanaal wordt getransloceerd, zou er een situatie kunnen ontstaan waarin het eiwit vast komt te zitten in het kanaal met een TM segment buiten de translocase en een periplasmatisch domein in het kanaal. Aangezien de efficiëntie van membraaneiwitinsertie door een gecrosslinked translocon vergelijkbaar is met die van een niet gecrosslinked translocon is het mogelijk dat het translocon/ribosoom/SecA complex anders omgaat met deze eiwitten als verwacht **(Fig. 2** D). Het is nog steeds niet duidelijk hoe het ribosoom eiwitten overdraagt aan SecA zodra een groot polair domein wordt aangetroffen. Wat zou er gebeuren als een membraaneiwit eerst een groot amino-terminaal periplasmatisch domein bevat en dan pas een carboxyl-terminaal TM domein? Hoe zou SecA met deze hydrofobe segmenten omgaan? Wat is de rol van het ribosoom in dergelijke processen, of vinden deze processen volledig post-translationeel plaats? Wanneer men denkt aan het brede scala aan eiwitten dat via het translocase geïnsereerd wordt in het membraan, wordt het duidelijk dat veel stappen in membraaneiwitbiogenese nog niet goed begrepen zijn. Nader onderzoek naar het mechanisme waarmee TM segmenten de laterale uitgang verlaten en de stappen waar het ribosoom, de translocase, SecA en andere ondersteunende eiwitten zoals YidC en SecDF(yajC) een rol spelen is daarom van groot belang. De ongehinderde TM insertie van een eiwit zoals MtlA door een translocase met een

gesloten laterale uitgang roept bijvoorbeeld vragen op over het mechanisme waarmee TM segmenten van SecYEG aan YidC overhandigd worden.

Koppeling van twee translocons aan de voorzijde heeft geen effect op eiwit translocatie of membraaneiwitinsertie

Diverse studies hebben gesuggereerd dat twee SecYEG translocons via de voorzijden ("voorzijde-aan-voorzijde") of achterzijden ("achterkant-aan-achterkant") een complex kunnen vormen. In een voorzijde-aan-voorzijde complex liggen de laterale uitgangen tegenover elkaar en zou bijvoorbeeld een samengevoegd kanaal kunnen ontstaan. Vanwege de resultaten zoals bescheven in **Hoofdstuk 3** is onderzocht hoe twee SecYEG translocons die via de voorzijden aan elkaar gekoppeld zijn fungeren. Om meer te weten te komen over de mogelijke rol van een "voorzijde-aan-voorzijde" complex in translocatie en membraaneiwitinsertie introduceerden we vlak naast de laterale uitgang een cysteïne. Door de cysteïnes van twee translocases een disulfide brug te laten vormen konden een stabiele "front-to-front" SecYEG complex gevormd worden. Theoretisch zou dit ons in staat moeten stellen te bepalen hoe en waar TM segmenten of signaalsequenties het translocon verlaten. Bovendien sluit deze manier van koppelen uit dat de twee translocons samen een kanaal vormen. De twee translocons konden efficiënt aan elkaar gekoppeld worden via cysteïnes in TM4 of in TM7. In beide gevallen had de koppeling geen effect op de translocatie activiteit, wat suggereert dat in een stabiele "voorzijde-aan-voorzijde" oriëntatie beide translocons actief zijn in translocatie. Onlangs werd gesuggereerd dat translocatie plaatsvindt door via de achterzijde gekoppelde SecYEG complexen, waarbij slechts een SecYEGs van het complex actief is in translocatie terwijl het

andere exemplaar een bindingsplaats voor SecA vormt [201]. Wij vonden echter dat een via de voorzijde gekoppelde SecYEG dimer even actief is in eiwittranslocatie als het wild-type of niet gekoppelde SecYEG complexen en dat hun activiteit net zo afhankelijk was van SecA. Bovendien vertoonden verdere in vitro eiwittranslocatie reacties aan dat een gekoppelde SecA dimeer op een efficiënte wijze werkt met het aan de voorzijde gekoppelde translocon en normale translocatieactiviteit vertoond. Hoewel we niet weten of beide delen van de SecA dimeer gelijktijdig actief zijn of na elkaar handelen, sluit deze waarneming aan bij eerdere studies waaruit bleek dat een dimere vorm van SecA van essentieel belang is voor eiwittranslocatie [64,122,123]. Tenslotte hebben we de rol van de ”voorzijde-aan-voorzijde” dimeer onderzocht in de insertie van membraaneiwitten, met FtsQ en MtlA als model substraten. Verrassend genoeg bleek de membraan insertie van zowel het SecA-afhankelijke FtsQ en het SecA-onafhankelijke MtlA normaal plaats te vinden via de voorzijde gekoppelde translocons. Hieruit blijkt dat een dimeer translocon ribosomen kan binden. Het moet echter opgemerkt worden dat het niet duidelijk of en hoe het ribosoom zichzelf boven de kanalen van beide translocons positioneert. Daarom is het waarschijnlijker dat het ribosoom alleen aan een van beide translocons bindt en het andere niet gebruikt. Onze gegevens suggereren dat de vorming van een gemeenschappelijk kanaal tussen twee via de voorzijde gekoppelde translocons niet plaatsvindt tijdens eiwittranslocatie of membraaneiwit insertie. Het is op dit moment nog niet duidelijk of er een specifieke rol is een SecYEG dimer die ofwel via de voor- of achterzijde zijn gekoppeld.

Toekomstperspectief

Terwijl we de afgelopen 20 jaar veel over het mechanisme van eiwittranslocatie en membraaneiwitinsertie te weten zijn gekomen, zijn er nog tal van vragen over. Brandende kwesties blijven bijvoorbeeld hoe transmembraan (TM) segmenten het translocase verlaten, hoe TM segmenten zich binnen het translocon heroriënteren en hoe deze tijdens membraaneiwitbiogenese van SecYEG naar YidC worden overgedragen. Ondanks alle experimentele resultaten die wijzen op de verschillende oligomere toestanden van het translocon en het motor-eiwit SecA, blijven de mechanistische rol(len) van deze toestanden onduidelijk. Ook de rol van andere ondersteunende eiwitten en complexen, zoals het SecDF(YajC) complex in zowel eiwittranslocatie en membraaneiwitinsertie is nog niet goed begrepen. In dit opzicht zullen eiwitten met niet essentiële, ondersteunende functies in deze processen experimenteel het lastigste te karakteriseren zijn. Nu er een aantal kristalstructuren van het translocon beschikbaar zijn kunnen gerichte benaderingen, zoals beschreven in dit proefschrift, worden gebruikt om het onderliggende mechanisme van eiwittranslocatie en membraaneiwitinsertie op te helderen. Bovendien kunnen de moleculaire mechanismen onderzocht worden op het niveau van individuele moleculen en dit kan gedetailleerd inzichten opleveren in de fysische en chemische werkingsmechanismen gedurende de verschillende stappen van de biogenese. Dit kan leiden tot nieuwe inzicht in de vorming van oligomere eiwitcomplexen en in het bijzonder de rol van YidC en andere eiwitten in de assemblage van deze complexen. Hoewel we steeds meer weten over in de structuur van SecYEG, is er nog relatief weinig bekend over YidC. Hiertoe zal eerst de structuur van YidC opgehelderd moeten worden.Terwijl we duidelijk een spannende tijden tegemoet gaan

in het onderzoek naar de structuur-functie relatie van het translocase, kunnen de in deze onderzoekingen nieuw ontwikkelde technieken ook worden gebruikt om andere membraantransportsystemen te bestuderen.

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